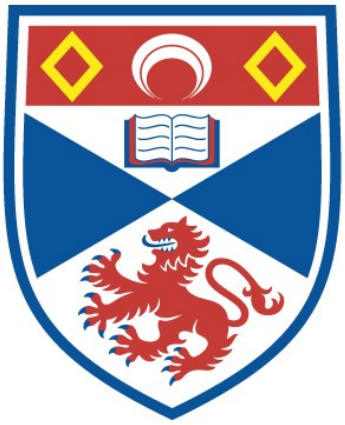
THE RETENTION OF DIFFERENTIATED FUNCTION IN CELL CULTURE : A STUDY OF THE UDCK CELL-LINE

Colin Douglas Archibald Brown

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE RETENTION OF DIFFERENTIATED FUNCTION IN CELL CULTURE:

A STUDY OF THE MDCK CELL-LINE.

A thesis submitted to the University of St. Andrews

for the degree of Doctor of Philosophy

-by-

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ABSTRACT

The transporting properties of MDCK cells have been investigated upon epithelial monolayers grown upon permeable filter supports and mounted in Ussing chambers. When mounted without edge damage epithelial monolayers of MDCK cells (strain I) exhibited a transepithelial electrical resistance of 7.9 kΩ.cm^2 and supported a small open circuit potential (5.9 mV, basal-lateral surface positive) and a small short circuit current in agreement with the small magnitude of the net Na^+, K^+ and Cl^- fluxes.

Addition of adrenaline to the basal-lateral, but not the apical bathing solution stimulated a net basal-lateral to apical Cl^- secretion, the magnitude of which accounted for the adrenaline stimulated short circuit current response. The Cl^- secretion in MDCK cells exhibited many of the features of Cl^- secretion in natural epithelia including sensitivity to the loop diuretics: furosemide, bumetamide and piretanide. In a number of secretory epithelia Cl^- is accumulated across the basal-lateral cell border by a diuretic sensitive Na+K+Cl cotransport system. A comparison of the actions of the loop diuretics upon adrenaline stimulated Cl secretion and upon the cotransport system in MDCK cells provided strong but indirect evidence for a central role of the cotransport system in Cl secretion across MDCK cell monolayers.

Measuremant of influx and efflux across the apical and basal-lateral cell border demonstrated that the apical cell border was relatively impermeable to K. Influx across the basal-lateral border consisted of three major compenents: a ouabain sensitive , pump mediated, component; a diuretic sensitive component and a ouabain and diuretic insensitive

flux component. K efflux across the basal-lateral cell border also consisted of these three components.

Addition of adrenaline, ATP or A23187 to MDCK cells resulted in a transient stimulation of K^+ efflux across both cell borders, although flux across the basal-lateral cell border was of greatest quantitative importance. Stimulation of K efflux was dependent upon the presence of Ca^{2+} in the external media and exhibited a similar pharmacology: sensitive to quinine and TEA, but insensitive to inhibition by apamin, as Ca^{2+}-dependent K permeabilities in other, natural epithelia.

Identification of an adrenaline stimulated Cl^- secretory mechanism, a coupled Na + K + Cl cotransport system and a Ca^{2+}-dependent K permeability in high resistance MDCK cells supports their use a model epithelium for the study of epithelial cell function.

Errata.

General.

For bumetamide read bumetanide.

For um and uM read μm and μM respectively.

Chapter 1.

Page 12, between lines 21 and 22 insert subtitle: (iii) Apical tight junctional morphology.

Page 14, line 13. Replace nM by nm.

Page 23, line 22. Delete: (Rabito) and replace with (Misfeldt & Sanders, 1981).

Page 28, line 3. Replace 'serosal to mucosal' by mucosal to serosal.

Chapter 2.

Page 36, line 17. Replace μM by μm.

Page 37, lines 19, 21 & 25. Replace μM by μm.

Chapter 3.

Figure legend 3.1, line 4. Replace absorpted by absorbed.

Chapter 4.

Page 87, line 1. Delete 'unity' insert 'close to unity'.

Chapter 5.

Page 106, line 25. Delete ' K^+ channels' insert ' Ca^{2+} channels '.

Page 113, line 27. Delete ' K^+ gating ' insert ' Ca^{2+} gating '.

Declaration

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is of my own composition. No part of this work has previously been submitted for a higher degree.

The research was conducted in the Department of Physiology and Pharmacology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr N.L. Simmons.

Academic Record

I first matriculated at the University of St. Andrews in October, 1975, and graduated in July, 1979, with the degree of B.Sc. Hons. Physiology (second class, upper division). I matriculated as a research student in the Department of Physiology and Pharmacology, University of St. Andrews in October, 1979.

Certificate

I hereby certify that Colin D.A. Brown has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Acknowledgements.

It is my pleasant duty to thank Dr. N.L. Simmons for his help, critism, discussion and enthusiastic support throughout the course of this study. I would also like to take this opportunity to thank Professor J.F. Lamb for extending the use of the facilities of the Department of Physiology & Pharmacology and for helpful discussion. Thanks must also go to members of staff and research students too numerous to mention for both encouragement and critical comment. For technical assistance, thanks are due to Mr I. Laurie, Mrs C. Voy and Mrs. M Falls. I owe grateful thanks to the Trustees of the Maitland-Ramsay Postgraduate Scholarship for generous financial support during this study.
There are two other people who deserve my special thanks: Mrs. Katrina Moss who accurately and speedily typed this manuscript and perhaps most of all my wife, Catriona, who not all devoted much time to the preparation of the figures in this manuscript, but also provided much needed encouragement and moral support over the last three years.

CONTENTS

SUMMARY

1. Epithelial monolayers of Madin and Darby canine kidney (MDCK) cells of between 60-74 serial passages (strain I) were grown upon permeable filter supports and mounted into Ussing type chambers for the measurement of their electrophysiological properties and transport properties.

2. Epithelial monolayers grown upon 2.5cm diameter filter supports exhibited a mean transepithelial electrical resistance of 3.5 $k\Omega.cm^2$ and supported a mean transepithelial potential of 1.2 mV (basal-lateral positive) and a small short circuit current (0.34 $uAmps.cm^{-2}$), consistent with the small magnitude of net tracer fluxes of Na^+, K^+ and Cl^-.

3. The effect of catecholamines upon the short circuit current and net tracer fluxes of Na^+, K^+ and Cl^- have been examined and compared to the effects of catecholamines upon ion transport in natural epithelia.

4. Addition of adrenaline to the basal-lateral bathing solution stimulated a significant increase in short circuit current as a result of an increased transepithelial potential and a decreased transmonolayer resistance. In contrast, application of adrenaline to the apical bathing solution was without effect upon short circuit current, implying that the receptors responsible for the response were located solely upon the basal-lateral cell membrane.

5. Measurement of net tracer Na^+, K^+ and Cl^- fluxes demonstrated that the adrenaline stimulated short circuit current was the result of a net Cl^- secretion from the basal-lateral to apical cell aspects. There was no significant increase in either Na^+ or K^+ fluxes. The magnitude of

the adrenaline stimulated Cl^- secretion was sufficient to account for the adrenaline stimulated short circuit current.

6. The adrenaline stimulated Cl^- secretion was dependent upon the presence of both Na^+ and K^+ in the basal-lateral bathing media. Na^+ could be replaced by Li^+ but not by choline. Cl^- could be replaced by Br^- but not by NO_3^-, I^-, SCN^- or isethiocyanate.

7. The pharmacological sensitivity of the adrenaline stimulated Cl^- secretion: sensitive to inhibition by the basal application of the loop diuretics: furosemide, bumetamide and piretanide, and to inhibition by phloretin from either bathing solution, but insensitive to inhibition by SITS or amiloride; was similar to the pharmacological sensitivity of secretagogue stimulated Cl^- secretion in natural epithelia.

8. The loop diuretics strongly inhibited adrenaline stimulated Cl^- secretion but had no effect upon the adrenaline mediated decrease in transmonolayer resistance.

9. Adrenaline, isoprenaline and noradrenaline all stimulated a significant increase in short circuit current. Half maximal stimulation of short circuit current was obtained with $1.5 \pm 0.2 \times 10^{-8}$M isoprenaline, $3.1 \pm 0.3 \times 10^{-8}$M adrenaline and $6.0 \pm 0.2 \times 10^{-6}$M noradrenaline, a potency order consistent with stimulation of a $beta_2$-adrenoreceptor.

10. Despite the high affinity of isoprenaline it was less effective in stimulating short circuit current than adrenaline. Analysis of the

adrenaline short circuit current with alpha- and beta-receptor agonists and antagonists implied that a substantial proportion of the adrenaline stimulated short circuit current was the result of an interaction of adrenaline with both alpha- and beta-receptors. The exact nature of the interaction is not clear.

11. In a number of Cl^- secretory epithelia a diuretic sensitive passive $Na^+ + K^+ + Cl^-$ cotransport system plays a central role in Cl^- accumulation across the basal-lateral cell membrane. To test whether this was the case in MDCK cells the properties of the cotransport system and adrenaline stimulated Cl^- secretion were compared.

12. When MDCK cell monolayers were grown upon large area monolayers (9.62 cm^2) and mounted in Ussing chambers designed to alleviate edge damage, the transepithelial electrical resistance was 7.9 ± 0.5 kΩ.cm^2 and the mean transepithelial potential was 5.9 ± 0.5 mV. Measurement of net Cl^- fluxes in the presence of adrenaline gave similar results as described above.

13. A comparison of the affinities of the ability of the loop diuretics to inhibit the cotransport system: bumetamide (K_i 0.4 ± 0.1 uM) $>$ piretanide (K_i 1.6 ± 0.4 uM) $\geq$ furosemide (K_i 1.6 ± 0.2 uM), and adrenaline stimulated Cl^- secretion: bumetamide (K_i 0.8 ± 0.1 uM) $>$ furosemide (K_i 2.5 ± 0.4 uM) $\geq$ piretanide (K_i 4.8 ± 0.3 uM) shows that they have similar affinities for the two processes.

14. The pharmacological sensitivity of the cotransport system: sensitive

to inhibition by furosemide: bumetamide, piretanide and phloretin, but insensitive to inhibition by SITS or amiloride; was identical to that for adrenaline stimulated Cl^- secretion.

15. The diuretic sensitive cotransport system exhibited an identical ionic dependence as adrenaline stimulated Cl^- secretion: the cotransport system was dependent upon the presence of both Na^+ and Cl^- in the basal-lateral bathing solution. Na^+ could be replaced by Li^+ but not choline and Cl^- could be replaced by Br^- but not by NO_3^-, I^-, SCN^- or isethiocyanate.

16. These results suggest that the $Na^+ + K^+ + Cl^-$ cotransport system plays a central role in Cl^- secretion across MDCK cell monolayers. The data also suggests that the mechanism of Cl^- secretion across MDCK cells is compatible with current models of epithelial chloride secretion in a number of natural epithelial tissues.

17. Measurement of influx and efflux across the apical and basal-lateral cell membrane demonstrated that the basal-lateral membrane was relatively impermeable to K^+.

18. Influx across the basal-lateral cell membrane consisted of three major components: a ouabain sensitive component, a ouabain insensitive but furosemide sensitive component, and a component of flux insensitive to inhibition by both ouabain and furosemide. The furosemide sensitive component was markedly dependent upon extracellular Cl^-.

19. Efflux across the basal-lateral membrane also consisted of a furosemide sensitive and a furosemide and ouabain sensitive component of flux. Ouabain caused a time dependent increase in the rate of K^+ loss from the cell.

20. A transient stimulation of K^+ efflux across either cell border was observed with the addition of adrenaline, ATP or A23187 to the bathing media. Adrenaline was only effective if added to the basal-lateral bathing media, ATP was effective from either solution and A23187 was effective only from the apical bathing solution.

21. Stimulation of K^+ efflux was dose dependent: half maximal stimulation of fractional K^+ loss with adrenaline was observed at 9.1×10^{-7}M. The actions of various adrenoreceptor agonists and antagonists were consistent with adrenaline action being mediated through an alpha adrenoreceptor. Half maximal stimulation of fractional K^+ loss with ATP was observed at 4.4×10^{-6}M.

22. Measurement of total cellular K^+ contents by flame photometry of tissue extracts indicated a net loss of K^+ in the presence of adrenaline. Similarly, adrenaline had no effect upon either the total K^+ influx or its distribution among the three flux components.

23. The stimulation of K^+ loss across either cell membrane by adrenaline, ATP or A23187 was abolished in a Ca^{2+}-free media.

24. The Ca^{2+} dependent K^+ permeability exhibited a similar pharmacology

to Ca^{2+} dependent K^+ permeabilities in other tissue: sensitive to inhibition by quinine and TEA but insensitive to the bee venom, toxin apamin.

25. Identification of an adrenaline stimulated Cl^- secretory mechanism, a $Na^+ + K^+ + Cl^-$ cotransport system, and a Ca^{2+} dependent K^+ permeability upon high resistance MDCK cell monolayers strongly supports the use of MDCK cells as a model epithelium with which to study epithelial cell function.

CHAPTER 1

GENERAL INTRODUCTION

A REVIEW OF THE RETENTION OF

DIFFERENTIATED FUNCTION IN CELL CULTURE

In recent years a powerful yet simple alternative approach to the study of epithelial cell function has emerged with the application of tissue culture techniques and the development of epithelial cell-lines that retain many of the features of the tissues from which they were derived (Handler et al, 1980a; Wright, 1981). In particular, rapid advances have been made in our understanding of the physiology (Cereijido et al, 1978; Simmons, 1981a; Misfeldt & Sanders, 1981; Bisbee, 1981; Perkins & Handler, 1981), pharmacology (Ludens et al, 1978; Roy et al, 1981; Simmons, 1981b; Rugg & Simmons, 1982) and cellular organisation of a wide range of cultured epithelia (Misfeldt et al, 1976; Peratoni & Berman, 1979; Handler et al, 1979; Cereijido et al, 1980a) which have remained differentiated in vitro. The aim of this introduction is to serve as brief review of the rapidly expanding literature upon the retention of differentiated function by epithelial cells in culture and to examine the usefulness of cultured epithelial cells as model systems with which to study epithelial function.

(A) The Retention of Epithelial Polarity in Cell Culture.

(i) Essential Features.

The ability of epithelia to perform directional transport of solutes and fluid is generally considered to be a direct consequence of two fundamental properties of epithelial cells: First they exhibit a striking morphological polarity; the plasma membrane appears, under microscopic study, to be composed of two discrete membrane domains - the apical and basalateral membranes which are sharply delineated by the junctional complex (see below). The morphological asymmetry of the plasma membrane into two domains has subsequently been demonstrated

to be the result of the functional polarity endowed to epithelia by a similar asymmetric distribution of enzymes, transport proteins, ion channels and hormone receptors to each of the two plasma membrane domains (Ebel et al, 1975; Kinne & Kinne-Saffran, 1981; Kenny & Maroux, 1982). The second fundamental property of epithelial cells is their ability to form sheets of similarly orientated cells, held together near the apical face by tight junctions (zonulae occludentes) which afford an effective permeability barrier to the free diffusion of the two bulk solutions, which may be of differing composition, across the epithelium (Erlij & Martinez-Palomo, 1978). A retention of these two features is a prerequisite for a cultured epithelium if it is to perform transepithelial transport.

Madin Darby Canine Kidney (MDCK) cells, derived from the kidney of an adult female Cocker-spaniel in 1958 by S.H. Madin and N.B. Darby and characterised by Gaush and co-workers (1966), provide strong evidence that they remain polarised in culture from the appearance of their plasma membrane. It is clear from many electron microscopic studies that the plasma membrane of MDCK cells is composed of two distinct membrane domains (Misfeldt et al, 1976; Cereijido et al, 1978; Barker & Simmons, 1981). Thus the uppermost membrane face (facing the media) is interspersed with microvilli, characteristic of the apical membrane of many natural epithelia (Kenny & Maroux, 1982), whilst the membrane face in contact with the substratum is free from any such protrusions, and has been designated the basal lateral membrane by several groups. Each cell is separated from its neighbour by a lateral interspace, characterised by extensive lateral inter-digitations of apposing membrane processes (fig 1.1). Each lateral space is sealed at, or near,

the apical membrane by a junctional complex (see below). A similar asymmetric morphology has been reported for several other cell-lines including LLC-PK_1, an epithelial cell-line derived from pig kidney, (Hull et al, 1976; Mills et al, 1979), TB-M and TB-6c, derived from toad urinary bladder (Handler et al, 1979), A6 cells, derived from toad kidney (Perkins & Handler, 1981) and several established or primary cell-lines derived from mammary epithelium (Emmerman & Pitelka, 1977; Bisbee, 1981). It should be noted however that many cell-lines originally derived from epithelial tissue do not retain junctional complexes or a polarised morphology in culture and therefore can be considered to have de-differentiated (for example; LLCPT, potoroo kidney epithelial cells (Zerban & Franke, 1978).

(ii) Plasma Membrane Protein Distributions.

As one might expect, the morphological asymmetry of the plasma membranes is reflected in an asymmetric distribution of membrane bound proteins and thus to different protein compositions of each membrane domain (Fujita et al, 1973; Ebel et al, 1975). For MDCK cells an asymmetric distribution of membrane proteins has been demonstrated by Richardson & Simmons (1979) using a lacto-peroxidase-mediated iodination of exposed membrane polypeptides at either face of epithelial monolayers of MDCK cell grown upon permeable filter supports. Furthermore a polarised distribution of specific apical and basal-lateral membrane marker enzymes has been reported by several groups. Thus, $(Na^+ + K^+)$-ATPase, generally regarded to be as a marker enzyme for basal-lateral membranes of all epithelia (Schmidt & Dubach, 1971; Stirling, 1972; Erlij & Ussing, 1978; Dibona & Mills, 1979), with the exception of

choroid plexus (Wright, 1978), has been shown to be restricted to the basal-lateral membrane face of MDCK cells by Louvard (1980), using an immunofluorescent localisation of a ($Na^+ + K^+$)ATPase antibody (Kyte, 1976) and in our own laboratory using an autoradiographic visualisation of ^{3}H-ouabain binding sites (Lamb et al, 1981). It is interesting to note that the results of the autoradiographic localisation suggest that the ($Na^+ + K^+$)-ATPase in MDCK cells is not evenly distributed across the basal (attachment) membrane but is almost exclusively located upon the lateral membrane face. Such an asymmetric distribution of pump sites has not previously been reported. 3H-ouabain autoradiography has also been used to demonstrate that the ($Na^+ + K^+$)-ATPase sites in LLC-PK_1 are also restricted to the basal-lateral membrane (Mills et al, 1979). Louvard has also demonstrated that leucine aminopeptidase, an apical membrane marker enzyme in both kidney and intestine (Vannier et al, 1976; Kenny & Maroux, 1982), is located solely upon the apical membrane face of MDCK cells.

However, despite several preliminary studies (Louvard, 1980; Richardson et al, 1981; Lamb et al, 1981), a biochemical dissection of plasma membrane protein distributions in cultured cells, as has been reported for natural epithelia (Fujita et al, 1973; Ebel et al, 1975; Vannier et al, 1976; Kenny & Maroux, 1982), has not yet been obtained.

It has long been recognised that the apical tight junction formed by the close apposition of the plasma membranes of neighbouring cells endowed epithelia with the ability to limit the free diffusion of solutes and fluids between the two bulk solutions they separate and combined with the transport properties of the epithelia modify the composition of either solution. Morphologically junctional complexes

are composed of a tight junction and associated desmosomes, the tight junction acting as the seal whilst the desmosomes hold the apposing membranes in close contact. The junctional complexes of MDCK cells are similar (fig 1.2 and Cereijido et al, 1978), desmosomes are also occasionally found deeper in the lateral interspaces, connecting apposing membrane processes. With the development of freeze fracture electron microscopy, a technique in which frozen plasma membranes are split along the central plane of the lipid bilayer to reveal the two internal membrane facets, designated the P-face and the E-face, corresponding to the interior and exterior half of the lipid bilayer respectively (Pinto da Silva & Branton, 1970; Branton et al, 1975) the structure of the tight junctions could be studied in greater detail. In both natural and cultured epithelia, junctional complexes were shown to be composed of variable numbers of strands made from collections of intramembranous particles (IMPs), generally considered to be protein, upon the P-face of the freeze-fracture (Chalcroft & Bullivant, 1970; Staehelin, 1973, 1974; Misfeldt et al, 1976; Cereijido et al, 1978). In some natural epithelia the number of junctional strands correlates well with the electrical resistance of the epithelium (Oschman, 1978; Claude & Goodenough, 1973), although this is not a universal feature (Martinez-Palomo & Erlij, 1975). The junctional complex is also thought to play a role in the maintenance of epithelial polarity: In toad urinary bladder cells, dissociation of junctional complexes is accompanied by the loss of the characteristic distribution of the "fuzzy coat" and ^{125}I-iodinated membrane proteins, normally restricted to the apical membrane, were seen to be evenly distributed across both membrane domains after only two hours (Pissam & Ripoche, 1976). On the

basis of these results the authors proposed that the tight junctions play an important role in the maintenance of membrane heterogeneity by preventing apical membrane proteins from migrating laterally into the basal membrane domain. Similarly the development of membrane polarity in MDCK cells is closely linked to the appearance of tight junctions in aggregating monolayers (Sang et al, 1980). In the past year an alternative view of the nature of tight junctions has emerged with the development of improved freezing techniques. Kachar and Reese (1982) have proposed a model for tight junctions based on the observation that membrane lipids and not intrinsic membrane proteins are the principle structural elements of the tight junction. Using unfixed material and a rapid freezing technique they observe pairs of cylinders(9-11 nM in diameter) which correspond to tight junction fibrils. They suggest that these cylinders are inverted lipid micelles, and not proteinaceous, and that the process of tight junction formation is akin to membrane fusion (Cullis & Hope, 1978). The model of lipidic tight junctions is supported by the work of Dragsten and colleagues (1981) that fluorescent lipid membrane probes can only cross from the apical to basal-lateral membrane domain of MDCK cells if they are capable of flipping from the E-face of a bilayer to the P-face.

Until comparatively recently the tight junction was considered as a sealed barrier to transepithelial solute and fluid movement. However it is now realised that in at least one group of epithelia the tight junction and lateral space is an important paracellular route for transepithelial movements of ions, small molecules and fluid. That the tight junction is the site of a conductance pathway was determined

using a voltage scanning technique (Fromter, 1972). This technique involved measuring the potential drop across an epithelium, generated by a constant current source, whilst moving one of the potential-sensing microelectrodes across the epithelium. The results indicated that the tight junction was the source of a high conductance pathway. Similarly the electron dense lanthanum ion has been used as a probe of junctional conductance (Machen et al, 1972; Tisher & Yarger, 1973, 1975). Transepithelial electrical resistance appears to be determined by the size of the junctional conductive pathway (see section (b1) below). The molecular mechanism by which transjunctional conductance is determined is as yet unclear. As we have previously said, in some epithelia there is a correlation between the number of junctional strands and the transepithelial resistance. In view of the lipidic model of tight junctions it would seem that transjunctional movement of polar molecules would be dependent upon either discontinuities along junctional strands or transport proteins straddling the lipid bilayer.

(B) Transepithelial Transport by Epithelial Cells in Culture.

As I have previously stated the ability of an epithelium to perform net transepithelial transport of solutes and fluid is determined by two main considerations; an asymmetric distribution of transport proteins and membrane permeabilities and the ability of an epithelium to provide an effective and selective permeability barrier to the movement of the bulk solutions. On the basis of the evidence presented in section (A) above, and especially the asymmetric distribution of the $(Na^+ + K^+)$-ATPase to the basal-lateral membrane domain, it would appear that several cultured epithelia may possess the ability to transport

solutes and fluid in a similar manner to natural epithelia.

(1) Indirect Measures of Transepithelial Transport in Cultured Epithelia

The first indication that cultured epithelia could perform transepithelial transport was the observation of fluid filled blister-like structures in confluent monolayers of cultured epithelia from such diverse origins as; kidney (Leighton et al, 1969; Hull et al, 1976; Handler et al, 1981), liver (Owens et al, 1974), thyroid (Ambesi-Impiombato et al, 1980), urinary bladder (Handler et al, 1979) and mammary epithelium (McGrath & Blair, 1970).

It is now generally accepted that blister formation is the result of an active accumulation of solute and fluid in the plane between the basal-lateral membrane face and the substratum. The resultant rise in hydrostatic pressure is thought to cause localised regions of the epithelial monolayer to lift from the substrata to form the characteristic fluid filled blister. For MDCK cells this supposition is based upon the following experimental evidence: blister formation is a dynamic process - by time-lapse cinematography blister turnover makes the epithelium resemble "gently boiling oatmeal" (Leighton et al, 1970). Blister formation can be inhibited by processes that block active transport such as ouabain and cooling the monolayer to $4^{\circ}C$ (Abaza et al, 1974). Blister formation can be enhanced by raising intracellular cAMP levels (Valentich et al, 1979; Lever, 1979), procedures which increase fluid reabsorption in natural epithelia (Grantham & Burg, 1966). Finally, blister formation is only seen when cells are grown upon impermeable supports. No blister formation has yet been observed in monolayers grown upon permeable, collagen

coated, nylon disks (Cereijido et al, 1978) or upon millipore filters (Misfeldt et al, 1976; Rabito et al, 1978).

Although at first sight blister formation may appear to be an ideal method of studying transepithelial transport in culture, and has been used as such by several groups (Valentich et al, 1979; Lever, 1979), it is an indirect measure of transport and as such has several drawbacks. Dome formation can be modified by factors which are not directly involved in transport such as changes in cell-cell and cell-substrate attachment (Handler et al, 1980) or the state of confluency of the monolayer (Valentich et al, 1979). Similarly, difficulties in interpreting blister formation in terms of transport events is further illustrated by the work of two groups. Rindler and co-workers (1979) reported that a MDCK sub-line which did not form blisters in culture exhibited an identical morphological, biochemical and hormone-sensitive adenylate cyclase profile as a blister-forming cell-strain. In our own laboratory we have a MDCK cell-strain (strain I) (Richardson et al, 1981) in which cAMP is implicated in hormone stimulated Cl secretion from basal to apical cell surfaces. These two examples suggest that although blister formation may be used as an initial indicator of transport ablility its applications to the study of transport phenomena is limited.

(2) Direct Measurement of Transepithelial Transport in Culture

A major advance in our knowledge of the physiology of cultured epithelia came with the application of the short circuit technique (Ussing & Zerahn, 1951) coupled with flux ratio analysis (Ussing, 1949) to cultured epithelial monolayers grown upon permeable supports and

mounted into Ussing chambers (Misfeldt et al, 1976; Misfeldt & Sanders, 1981; Bisbee, 1981; Handler et al, 1981). These techniques have previously been used to elucidate transport processes in a wide range of natural epithelia (Ussing & Zerahn, 1951; Schultz & Zalusky, 1964; Zadunaisky, 1966; Wright, 1972; Widdicombe & Welsh, 1980).

(a) Measurement of Transepithelial Resistance: On the basis of transepithelial resistance, epithelia can be sub-divided into two major groups (Rose & Schultz, 1971; Fromter & Diamond, 1972). The main determinent of epithelia resistance appears to be the ability of the apical tight junction to form an effective permeability barrier to the movement of ions and small molecules, i.e. junctional tightness, (Fromter, 1972; Machen et al, 1972; Erlij & Martinez-Palomo, 1978). The first group of natural epithelia are characterised by low transepithelial electrical resistance (5 - 400Ωcm^2) and generate only small spontaneous electrical potentials. In these epithelia a large part of the passive transepithelial solute and fluid movement occurs via an extra-cellular shunt pathway across the tight junction, (Rose & Schultz, 1971; Frizzell & Schultz, 1972). Included in this group are epithelia from as diverse origins as: renal proximal tubules (Boulpaep & Seely, 1971), rabbit ileum (Frizzell & Schultz, 1972), choroid plexus (Wright, 1972) and gallbladder (Fromter, 1972).
The second group of epithelia, in contrast, are characterised by high transepithelial electrical resistances (up to 10 000Ωcm^2) and in most cases generate large spontaneous potentials. In these epithelia most transepithelial solute movement is via a transcellular route, the paracellular shunt accounting for less than 10% of the total trans-

epithelial flux in some epithelia (Erlij & Martinez-Palomo, 1978). Epithelia classed as being "tight" epithelia include: renal distal (Wright, 1971; Boulpaep & Seely, 1971) and collecting tubules (Helman et al, 1971), amphibian urinary bladder (Erlij, 1976) and frog skin (Mandel & Curran, 1972; Helman & Miller, 1973).

It is perhaps not surprising that a similar diversity of transepithelial resistance values are displayed by differentiated epithelial cells in culture (table 1.1). Using the criteria discussed above, the renal cell-line LLC-PK_1 and primary cultures of mammary epithelia can be classed as "leaky" epithelia, whilst the cell-lines derived from urinary bladder, TB-M and TB-6c, and from toad kidney (A6) may be classed as tight epithelia. The MDCK cell-line however presents two separate electrical profiles. On the one hand several groups have reported that MDCK cells exhibit many of the features normally associated with natural "leaky" epithelia: These cells exhibit a low transepithelial resistance, in the range 80 - 173 Ωcm^2, and generate a small spontaneous electrical potential of about 1 mV, apical surface negative. The tight junctions are, as is common in natural leaky epithelia, (Machen et al, 1972; Tisher & Yarger, 1973), permeable to lanthanum ions (Barker & Simmons, 1978; Cereijido et al, 1980a) and are cation selective (Misfeldt et al, 1976; Cereijido et al, 1978; Rabito et al, 1978; Barker & Simmons, 1981). The junctional selectivity of low resistance MDCK cells to ions ($K > Na > Li >$ choline and $NO_3 > CL \gg I >$ isethionate) is similar to the ion selectivity of junctions of natural leaky epithelia (Barry et al, 1971; Fromter, 1979). The second group of MDCK cells form monolayers with high electrical resistances (table 1.1) but unlike many tight epithelia only generate a small

electrical potential. It has been suggested that the transepithelial resistance of these monolayers has, as the result of crush damage incurred in mounting the monolayers into Ussing chambers (Helman & Miller, 1973), been underestimated, and that the true resistance of an undamaged monolayer is in the region of 8 kΩ cm^2 (Barker & Simmons. 1981). Consistent with natural high resistance epithelia tight epithelial monolayers of MDCK cells do not have an appreciable para-cellular shunt pathway; indicated by the lack of dilution potentials or bi-ionic potentials and by the failure of apically applied lanthanum to cross the junctional complex (Barker & Simmons, 1981). At first it was suggested that the electrical properties of MDCK cells were dependent upon the number of serial passages from origin a culture was, since cells of 60-74 serial passages formed "tight" monolayers, whilst cells of between 110-120 passages formed leaky epithelial monolayers (Barker & Simmons, 1981 and table 1.1). Subsequent studies however suggest that several strains (or sub-lines) of MDCK cells exist which can be distinguished by their morphology, electrical properties and enzyme profiles: high resistance MDCK cells are smaller and have fewer microvillae than low resistance MDCK cells (Barker & Simmons, 1981). Biochemically: high resistance MDCK cells lack any measurable alkaline phosphatase or γ-glutamyl-transpeptidase activity and have only half the activity of ouabain-insensitive ATPase activity of low resistance MDCK cells (Richardson et al, 1981). Cereijido and co-workers (1981) have also reported that MDCK cells of below 65 serial passages can also exhibit a low transepithelial resistance.

(b) The Nature and Magnitude of the Spontaneous P.D. Generated by Epithelial Cells in Culture: The potential generated by epithelia, under conditions free from electrochemical gradients, is due to a net movement of charge across the epithelia as the result of active transport. In most natural epithelia the spontaneous potential is the result of net Na^+ absorption generated by the asymmetric distribution of the $(Na^+ + K^+)$-ATPase and the permeability properties of each membrane face (Ussing & Zerahn, 1951; Ussing & Leaf, 1978). For cultured epithelia there is both direct and indirect evidence in favour of a major contribution from active Na^+ transport in the generation of spontaneous transepithelial potentials. Direct measurement of net Na^+ ion transport, using ^{22}Na and ^{24}Na as tracers of bidirectional Na^+ fluxes, have been reported for several cultured epithelia: in cell-lines derived from toad urinary bladder (TBM and TB-6c) and kidney (A6), which maintain spontaneous potentials of between 9-40 mV, apical negative, (table 1.1), the net apical to basal Na^+ flux can account for all the short circuit current although for TB-6c a small proportion of the short circuit current remains unaccounted for (Handler et al, 1979; Perkins & Handler, 1981). Similarly net Na^+ absorption accounts for all the short circuit current in cell-lines derived from mammary epithelia (Bisbee, 1981). Direct measurement of Na^+ flux across MDCK cell monolayers is more problematic, due to the small magnitude of the spontaneous potential (1-3 mV, apical negative) and to the resolution of the flux technique. Measurement of net flux across low resistance monolayers of MDCK cells is further complicated by large passive unidirectional Na^+ fluxes through the paracellular shunt pathway. Of the two groups to measure trans-

epithelial Na^+ fluxes across low resistance MDCK cell monolayers, one group reported a small (2.6 umol. $cm^{-2}.hr^{-1}$) apical-to-basal flux (Cereijido et al, 1980a), although this result is subject to large errors since only a single tracer technique was employed. The second group, using a double label technique, were unable to demonstrate any net Na^+ flux in either high resistance or low resistance MDCK cell monolayers (Barker & Simmons, 1981) although they reported that the unidirectional Na^+ fluxes across low resistance cell monolayers were of a similar order to those reported by Cereijido and co-workers (1980a). A major role for Na^+ ions in maintaining the spontaneous potential across MDCK and LLC-PK_1 cell monolayers is supported by the observation that the P.D. is sensitive to the application of ouabain to the basal bathing solution (Cereijido et al, 1978; Simmons, 1981a; Misfeldt & Sanders, 1981), coupled to the localisation of the (Na^+ + K^+)-ATPase to the basal-lateral membranes of MDCK and LLC-PK_1 cells (Louvard, 1980; Lamb et al, 1981; Mills et al, 1979).

In many epithelia the coupling of solute flux to an inwardly directed Na^+ flux plays an important role in the transepithelial transport of a wide range of organic and inorganic solutes from the mucosal to the serosal membrane face. In intestine: hexose transport (Crane, 1977), amino acid absorption (Hopfer et al, 1976), bile salts (Wilson & Treaner, 1979), inorganic phosphate (Berner et al, 1976) and chloride ions (Eveloff et al, 1980) are all transported across the apical membrane by a sodium dependent mechanism. A similar range of solutes also proceed by sodium dependent co-transport systems in the proximal tubules of the kidney (Ullrich, 1980; Fromter, 1979; Spring & Kimura, 1978). For solutes accumulated into the cell across the apical

membrane by sodium dependent processes, the exit step, across the basal-lateral membrane is usually passive, driven by its inherent electrochemical gradient (for a review see Alvarado, 1976).

In cultured epithelium a Na^+-dependent-hexose uptake mechanism has been reported in the pig kidney cell-line LLC-PK_1 (Mullins et al, 1979; Rabito & Aussiello, 1980; Misfeldt & Sanders, 1981). The transport system shares many features in common with sugar transport across both renal proximal tubules and small intestine: sugar accumulation across the apical border is dependent upon the presence of sodium ions in the apical bathing solution and upon a functioning ($Na^+ + K^+$)-ATPase on the basal-lateral membrane (Silverman, 1975; Alvarado, 1976). There is a good correlation between the sugar concentration in the apical bathing solution, the transepithelial potential difference and the short circuit current, although the latter two saturate at higher sugar concentrations (Misfeldt & Sanders, 1981), a similar correlation is observed between sugar concentration and short circuit current in natural epithelia (Schultz & Zalusky, 1964; Quay & Armstrong, 1969). The sugar transporter has a similar stereospecificity requirement and affinity sequence, measured either as the ability of a sugar to inhibit radio-labelled methyl-D-glucose uptake (Rabito & Ausiello, 1980) or to stimulate short circuit current (Rabito) both methods gave identical results as sugar transport across intestine (Barry et al, 1965) and renal proximal tubule (Ullrich et al, 1974). In an elegant series of studies Kinne and co-workers (1975) demonstrated that sugar accumulation into brush-border (apical) membrane vesicles is phlorizin sensitive, whereas sugar transport across basal-lateral membranes is phlorizin insensitive but phloretin

sensitive. In LLC-PK_1 cell epithelium both uptake of methyl-D-glucoside across the apical plasma membrane and the steady state short circuit current, in the presence of D-glucose, is inhibited by apically applied phlorizin basal application is without effect (Rabito & Ausiello, 1980; Misfeldt & Sanders, 1981). Furthermore phloretin actually stimulates cellular accumulation of methyl-D-glucose, presumably by inhibiting its passive movement across the basal-lateral membrane (Rabito & Ausiello, 1980). The conclusion drawn from these studies was that LLC-PK_1 cells retained in culture a functional hexose transport system which closely resembled those already described, and could provide a useful model system with which to study transepithelial sugar transport. It is worth noting that LLC-PK_1 cells do not appear to possess any of the other sodium dependent solute transport systems normally associated with either renal or intestinal epithelia (Misfeldt & Sanders, 1981).

(c) Hormonal Stimulation of Transepithelial Transport and Adenylate Cyclase Activity in Cultured Epithelia.

It is now generally accepted that the cellular actions of a variety of hormones are mediated via a membrane bound adenylate cyclase system consisting of a specific hormone receptor, a regulatory subunit and a catalytic moeity, with resultant changes in intracellular cyclic nucleotide levels (Sutherland & Rall, 1960; Hays & Levine, 1974; Goltzman et al, 1976; Katz & Lindheimer, 1977). In epithelia changes in intracellular cAMP levels are usually associated with changes in transepithelial solute and fluid transport (Grantham & Burg, 1966; Stoff et al, 1977; Field et al, 1975; Frizzell et al, 1979). Although

the hormone receptor and catalytic subunit are separate entities stimulation of adenylate cyclase activity has been extensively employed as a means to determine the distribution of hormone receptors in complex epithelia (Chabardes et al, 1975; Imbert et al, 1975; Morel et al, 1976; Bailly et al, 1980; Morel, 1981).

(1) MDCK Cells

An initial characterisation of the adenylate cyclase of MDCK cells (Rindler et al, 1979) reveals that it shares many features in common with adenylate cyclase units in other tissue: the adenylate cyclase of MDCK cells is dependent upon the presence of both GTP and Mg^{2+} ions in the incubation mixture for activation. GTP is known to interact with the regulatory subunit of the cyclase and its presence is crucial for enzymatic activation by hormones (Lefkowitz et al, 1976; Howlett et al, 1979). The regulatory role of Mg^{2+} ions upon the receptor-cyclase complex has only recently been recognised (for a review see Cech et al, 1980). It is thought that the adenylate cyclase complex has specific Mg^{2+} binding sites which when occupied can modulate the hormone receptor affinity and the maximal rate of catalytic action. In MDCK cells the optimal free Mg^{2+} concentration is 1mM - similar to the optimal concentration for activation of the catalytic subunit of S49 lymphoma cells (Cech et al, 1980). The hormonal sensitivity of the adenylate cyclase; vasopressin, prostaglandins ($E > A \gg F \gg B$), oxytocin, glucagon and isoprenaline but not to parathyroid hormone or calcitonin (Rindler et al, 1979; Mita et al, 1980) agrees well with the hormonal sensitivity of whole cells(Ishizuka et al, 1978). It has been argued that since the hormonal sensitivity of the adenylate cyclase of MDCK

cells mirrors that of the distal (connecting) and collecting tubule of rabbit kidney (Bailly et al, 1980; Morel, 1981) coupled with the lack of proximal tubule "marker" enzymes (Rindler et al, 1979) and a relatively high transepithelial resistance, in comparison with proximal renal tubule, (Barker & Simmons, 1981) that MDCK cells may have originally stemmed from distal/collecting tubule cells. The physiological correlate of increases in intracellular cAMP levels may be the stimulation of transepithelial transport. In this context Richardson and colleagues (1981) reported that vasopressin, prostaglandin E_1 and adrenaline (which has similar actions to isoprenaline) all stimulated short circuit current, and therefore transport, in tight epithelial monolayers of MDCK cells. The short circuit current stimulated by PGE_1 was the result of active Cl^- secretion (Simmons, 1979). Similarly cAMP is known to stimulate blister formation in confluent MDCK cell cultures (Valentich et al, 1979; Lever, 1979), although the physiological mechanism remains obscure (Handler et al, 1980).

The mechanisms by which cAMP regulates cellular function are not yet fully understood although it would appear to be likely that a cyclic AMP- dependent protein kinase may be one of the major pathways by which a hormonal signal is transduced into a cellular response (Nimmo & Cohen, 1977; Glass & Krebs, 1980; Cohen, 1982). A role for a protein kinase in mediating the cellular effects of a vasopressin induced rise in intracellular cAMP levels has recently been proposed by Mita and co-workers (1980).

(2) LLC-PK_1 (Pig Kidney Epithelial Cells)

A hormone sensitive adenylate cyclase has been identified in LLC-PK_1

cells (Goldring et al, 1978). An increase in intracellular cAMP levels can be elicited by incubating the cells with lysine and arginine vasopressin, pitressin and calcitonin but not with adrenaline, parathyroid hormone or prostaglandin PGE_2. On the basis of the hormonal sensitivity of the adenylate cyclase the authors postulated that LLC-PK_1 cells may have derived from the medullary portion of the thick ascending limb of the loop of Henle. These results were subsequently verified by Roy and his co-workers (1980). This group was also the first to correlate hormone stimulated adenylate cyclase activity with hormone receptor occupancy in a cultured epithelial cell-line. Using a ^3H-labelled lysine vasopressin moiety they demonstrated that the time course, receptor affinity (K_D 10nM) and the ability of other vasopressin analogues to displace ^3H-vasopressin binding are identical to those previously reported for plasma membranes isolated from pig renal medulla (Bockaert et al, 1973; Roy et al, 1975). Using a subline of LLC-PK_1 adapted to growth in a serum-free, chemically defined media the group was also able to show that vasopressin receptor numbers and adenylate cyclase activity could be increased, from 5% of the parent cell-line, by including both 10% foetal bovine serum and insulin in the growth media. However factors which result in modulations in receptor numbers in vivo such as hydrocortisone, dexamethasone and thyroid hormone (Rajerison et al, 1974; Williams & Lefkowitz, 1979; Harkom et al, 1978) are without effect upon vasopressin receptor numbers in LLC-PK_1 cells. Thus it would appear that foetal bovine serum contains some other factor which stimulates the production of vasopressin receptors. The physiological function of these receptors has not yet been defined.

(3) Other Cell-lines

The three cell-lines derived from amphibian epithelia (TB-M and TB-6c from toad urinary bladder and A6 from toad kidney) transport sodium from the serosal to mucosal membrane face and this net transport is equivalent to the resting short circuit current in TB-M and TB-6c, but slightly less than the short circuit current for A6 cells. Sodium transport and short circuit current can be increased by aldosterone in all three lines. In TB-M cells the aldosterone stimulated sodium transport is also associated with a decrease in transepithelial resistance (Handler et al, 1979; Perkins & Handler, 1981). In intact epithelia aldosterone stimulates Na^+ transport by a process that involves protein synthesis. The mechanism of action by which aldosterone acts to increase Na^+ transport is as yet unclear but is thought to involve either the synthesis of apical sodium channels or a stimulation of Na^+ efflux across the basal-lateral border, or a combination of the two events (Finn, 1978). On the basis of the differing responses of these cells to aldosterone Handler and colleagues have speculated that Na^+ transport across TB-M cells was associated with an increase in apical membrane Na^+ channels, hence the increase in transepithelial conductance, whereas the response of TB-6c and A6 cells lacked this mechanism and Na^+ transport was the result of an increased flux across the basal-lateral membranes (Handler et al, 1980a; Handler et al, 1981). Vasopressin, the major hormone acting on transepithelial sodium and fluid transport in a number of epithelia including amphibian urinary bladder and mammalian renal tubules (Hays & Levine, 1974; Hebert et al, 1981), is surprisingly without effect upon Na^+ transport, intracellular cAMP levels or water permeability

(Handler et al, 1981). Although no adenylate cyclase activity could be stimulated by vasopressin, prostaglandin E, fluoride and guanine nucleotides were all effective stimulators of adenylate cyclase activity in broken cell preparations. Similarly synthetic analogues of cAMP were able to stimulate net Na^+ absorption (Perkins & Handler, 1981), urea permeability and short circuit current (Handler et al, 1981), but had no effect upon transepithelial water permeability. Application of amiloride to the apical bathing solutions results in a diverse range of responses from these epithelia: amiloride inhibits the basal, the aldosterone-stimulated and the cAMP stimulated short circuit current in A6 cells; the basal and the aldosterone-stimulated short circuit current in TB-M and only the aldosterone-stimulated short circuit current in TB-6c cells. The reason for these diverse responses is not clear.

Hormonal regulation of net sodium transport has also been reported for primary cell cultures derived from mouse mammary epithelium. Under steady state conditions these cells maintain a short circuit current of 25 $uA.cm^{-2}$ which can be accounted for by the net sodium transport (absorption) (Bisbee, 1981). Addition of prolactin to the bathing media results in a fourfold increase in net sodium absorption, a decrease in transepithelial resistance and a decrease in net basal to apical sucrose transport. The increase in Na^+ movement is twice what can be accounted for by the increase in short circuit current, suggesting that prolactin stimulates the rheogenic transport of some other ionic species. Increases in Cl^- and K^+ fluxes have been ruled out (Bisbee, 1981) but possible candidates are Ca^{2+} ions or H^+ ion secretion.

(C) Summary and Conclusions

From the evidence presented in this short review it is clear that cultured epithelial cells from as diverse origins as mammalian and amphibian kidney and mammalian mammary epithelia remain differentiated in culture, retaining typical morphological, biochemical and functional characteristics of their tissues of origin. However at this point it may be timely to point out that these cells are adapted to growth under culture conditions and as such are unlikely to exhibit exactly the same characteristics as *in vivo*. To illustrate this point the pig kidney cell-line LLC-PK_1 exhibits a sodium dependent sugar transport mechanism (Rabito & Ausiello, 1980; Misfeldt & Sanders, 1981) and an enzyme profile normally associated with renal proximal tubules (Ullrich, 1980; Peratoni & Berman, 1979) yet displays a hormonal sensitivity of its adenylate cyclase akin to the medullary region of the thick ascending limb of the loop of Henle (Goldring *et al*, 1978) and a trans-epithelial electrical resistance (200-400 Ω cm^2) usually associated with distal tubular epithelium (Erlij & Martinez-Palomo, 1978). This does not however negate the usefulness of LLC-PK_1 as a model epithelium. In order to overcome objections raised as to the origins, in terms of nephronal segment, several groups are developing cultures from defined nephron segments (Horster, 1980; Richardson *et al*, 1982; Burg *et al*, 1982) although these studies are still in their infancy.

Thus, although it is clear from the literature that many epithelia remain differentiated in culture and have been used extensively to study the development and maintenance of epithelial polarity (Rodriguez-Boulan & Sabatini, 1978; Sang *et al*, 1980; Cereijido *et al*, 1980; Martinez-Palomo *et al*, 1980; Meza *et al*, 1980), very few studies have

exploited the inherent advantages in the use of a cultured epithelium to study transepithelial transport. Among these advantages are:

(1) Epithelial monolayers formed by epithelial cells in culture, upon either petri dishes or upon permeable supports, are one cell thick and so form the simplest epithelial structure possible in contrast to the complicated epithelial geometry of isolated renal tubules or the multi-layer composition of other epithelial preparations (Nellans et al, 1974; Frizzell et al, 1976; Erlij & Ussing, 1978).

(2) In contrast to in vitro preparations cultured epithelia remain viable for long periods of time at 37°C in Krebs buffer (see methods), thus allowing experiments of longer duration. Another advantage is that the chronic effects of an agent can be studied by including it in the growth media.

(3) One of the major problems with natural epithelia is gaining enough homogenous starting material for biochemical assays and for membrane vesicle preparation. With a cultured system large quantities of homogenous cells can be prepared, without resort to cell separation procedures (Scholer & Edelman, 1979), by simply increasing the size of the culture vessel.

The main aim of this thesis is therefore to investigate some of the transport properties of the dog kidney cell line MDCK, and to validate its use as a model system for the study of epithelial transport.

TABLE 1.1

A summary of transepithelial electrical resistances and open circuit transepithelial resistances for cultured epithelia grown upon permeable supports. The data is taken from the following works.

1. Misfeldt *et al*, 1976
2. Cereijido *et al*, 1978
3. Rabito *et al*, 1978
4. Barker & Simmons, 1981
5. Richardson *et al*, 1981
6. Simmons, 1981a
7. Misfeldt & Sanders, 1981
8. Handler *et al*, 1980a
9. Perkins & Handler, 1981
10. Handler *et al*, 1981
11. Bisbee, 1981
12. Burg *et al*, 1981

Tissue of origin	Cell-line or primary culture	P.D.(mV)	Resistance $\Omega.cm^2$	Ref.
Dog kidney	MDCK	0.8-1.4	84-104	1-3
Dog kidney	MDCK (strain I)	2.2	4116	4,5
Dog kidney	MDCK (strain II)	0.2	70	4,6
Pig kidney	$LLCPK_1$	2-2.7	211-400	7,8
Toad kidney	A6	9-10	6900-7020	9,10
Toad urinary bladder	TB-M	36.7	4680	10
Toad urinary bladder	TB-6c	20.5	9 290	10
Mouse mammary gland	primary	12.8	507	11
Rabbit thick ascending limb	primary	-9.9	-	12

Fig 1.1 Low power electron micrograph of an MDCK cell monolayer.

This micrograph shows the retention of polarised morphology by high resistance MDCK cells grown upon a permeable filter support (mp). The uppermost surface of the monolayer has sparse brush border with small microvillae (mv). Cells are joined at the apical surface by tight junctional complexes (tj ; see also fig 1.2). Between adjoining cells are lateral interspaces (ls), characterised by extensive infoldings of the basal-lateral cell membranes.

MDCK-cells

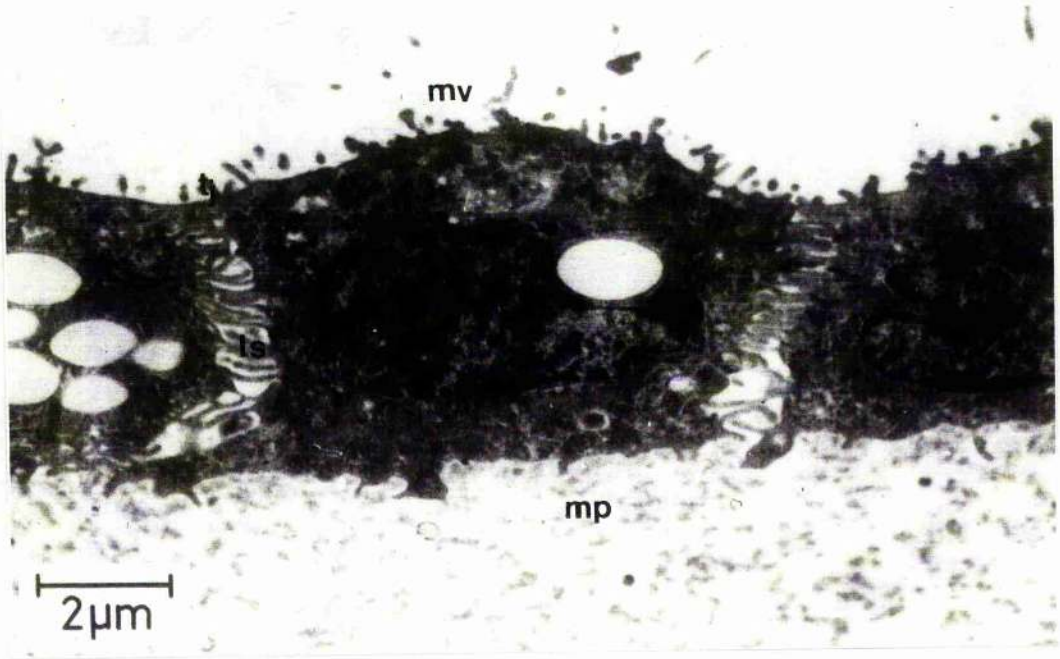
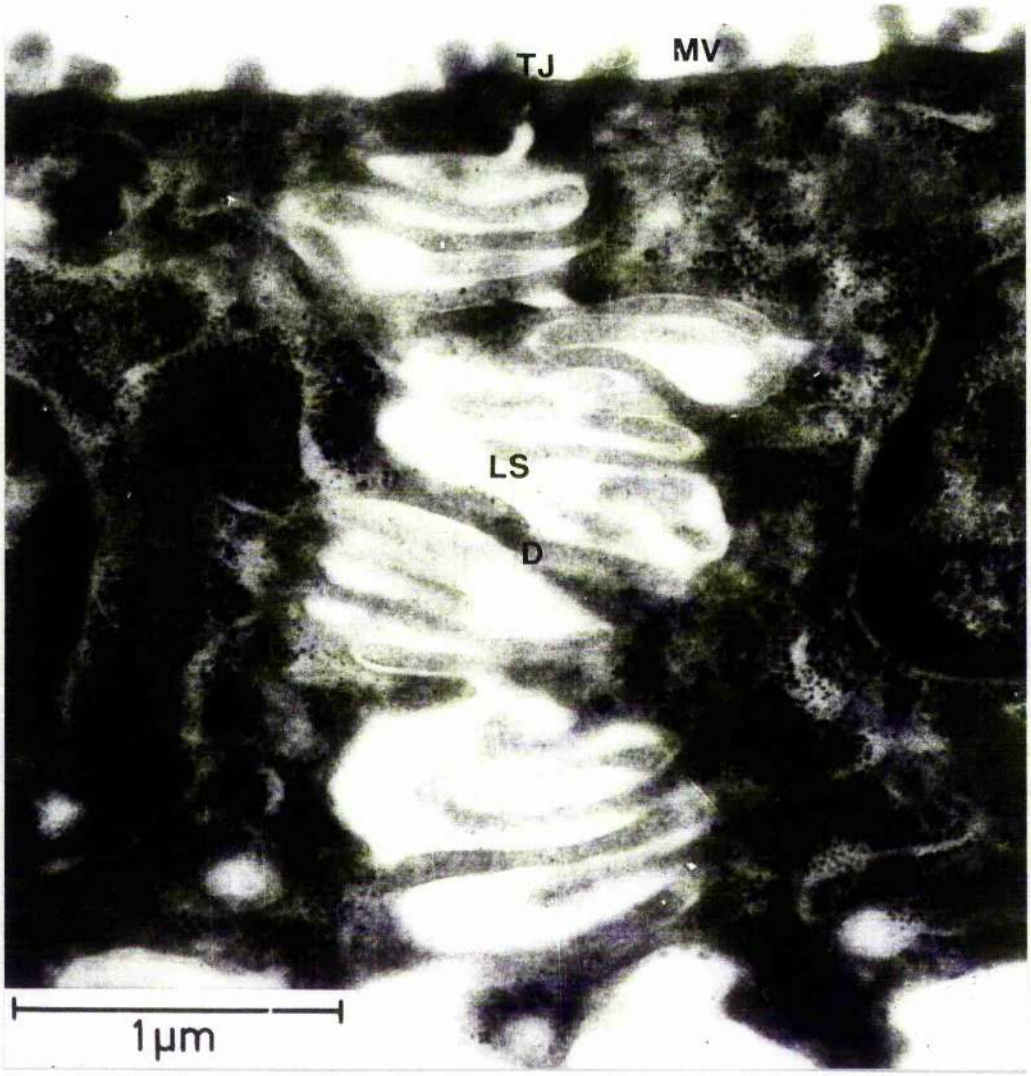


Fig 1.2 The junctional region of an MDCK cell monolayer.

A high power electron micrograph of the junctional complex and lateral space of an MDCK cell monolayer grown upon a permeable filter support. The tight junction is formed from a zonula occludentes and associated desmosomes (TJ). The lateral space (LS) contains numerous membrane infoldings some of which form physical contact with each other through desmosomes (D).

MDCK-cells



CHAPTER 2

MATERIAIS AND METHODS

MATERIALS

Cell Culture

All tissue culture cells and consumables, unless otherwise stated, were obtained from Flow Laboratories, Irvine, Scotland. Gentamycin antibiotic was supplied by Roussel Pharmaceuticals Ltd., London, U.K. Insulin, as sterile injections, was obtained from Boots Chemical Co. Ltd. Nottingham, U.K. Disposable plastic petri dishes (3cm, 5cm, 9cm diameter) were supplied by Sterlin Ltd., Teddington, U.K. The permeable filter supports were millipore filters (type GM, 0.22 uM pore size, 2.5 cm and 4.7cm diameter) from Millipore Corp., Harrow, U.K.

Radiochemicals

^{22}Na, ^{24}Na, ^{42}K and ^{86}Rb as aqueous solutions of the chloride salts and ^{36}Cl as an aqueous solution of the sodium salt were supplied by Amersham International, U.K. ^{77}Br in isotonic saline was purchased from The Medical Research Council's Cyclotron Unit at Hammersmith Hospital, London, U.K.

Chemicals and Biochemicals

All chemicals were of an ANALAR grade or equivalent, and were from B.D.H. Ltd., Poole, U.K. or from Fisons Ltd., Loughborough, U.K. In general biochemicals were obtained from Sigma Chemicals Ltd., Poole, U.K. unless otherwise stated. Phentolamine (Rogitine) was from Ciba-Geigy, Manchester, U.K. The stilbene; 4,-acetamido - 4',-isothiocyanato-stilbene - 2,2'-disulphonic acid (SITS) was from B.D.H. Ltd., Poole, U.K. The calcium ionophore A23187 was purchased from

Calibiochem Ltd., C.P. Laboratories, Bishops Stortford, U.K. Ethacrynic acid, amiloride and oxymetozaline were gifts of Merk, Sharp and Dohme, Hoddeson, Herts., U.K. Propranolol hydrochloride was a gift from I.C.I. (Pharmaceuticals), Macclesfield, U.K. The diuretics furosemide and piretanide were generously donated by Dr. S. Dombey of Hoescht, Hounslow, U.K. Bumetamide was obtained from Leo Pharmaceuticals, Ballerup, Denmark.

METHODS

(A) Cell Culture

All experiments described in this thesis were performed upon MDCK cells of either 66 - 74 serial passages or 110 - 120 serial passages. Cell cultures of either strain were initiated by reactivating cells from frozen stocks prepared in this laboratory and held in the vapour phase of a liquid nitrogen safe (Union Carbide, Darlington, U.K.). Cells had originally been supplied by Flow Laboratories, Irvine, Scotland, at 60 serial passages (April, 1978) and at 109 serial passages (November, 1976). Both cell strains were grown under identical conditions in Minimum Essential Media Eagle's with Earle's salts and 0.85 $g.l^{-1}$ $NaHCO_3$ (Table 2.1) supplemented with 10 (v/v) foetal bovine serum, 1% (v/v) non essential amino acids and 1 $unit.cm^{-3}$ gentamycin antibiotic at 37°C in a 95% / 5% air/CO_2 atmosphere. In later experiments, because of difficulties in obtaining foetal bovine serum, 10% FBS was replaced in the media with 5% (v/v) FBS and 5% (v/v) donor horse serum. This change had no effect upon any of the parameters measured.

(i) Propagation and Maintenance of MDCK Cell Cultures.

MDCK cells were routinely grown in Roux flasks (120 cm^2 growth area) and for experimental purposes as monolayers on plastic petri dishes or upon permeable filter supports. Confluent Roux flasks were subcultured (passaged) at weekly intervals. To prepare a cell suspension from a confluent Roux flask the growth media was removed and the monolayer rinsed with 5 cm^3 of a trypsin solution (0.25% trypsin in a Ca^{2+}/Mg^{2+}-free Earle's salt solution with 2 mM EDTA). A further 5 cm^3 of trypsin solution was then added and the monolayer incubated at 37°C until the

cells detached from the substratum. The enzymic action of trypsin was neutralised by the addition of 45 cm^3 of complete growth media - any remaining cell clumps were broken up by repeated (x 10) syringing of the cell suspension through a wide bore needle (1mm diameter). Cell number and an estimate of cell volume were determined from a 1 cm^3 sample of the cell suspension using a Coulter Counter (model ZF) with a channelyser attachment (model C1000) (Boardman et al, 1974). The culture was continued by seeding a fresh roux with 5 x 10^6 cells, and the remaining cells were used to seed permeable filter supports or petri-dishes for experimental purposes.

(ii) Estimation of Cell Numbers and Cell Volume.

Cell number and mean cell volume of 1 cm^3 aliquots of cell suspension prepared by trypsinisation diluted with 19 cm^3 of Isoton, a commercially available isotonic electrolyte solution, were estimated using an electronic particle sizer and associated 100 channel pulse height analyser (Coulter Electronics, Dunstable, U.K.).

Cell size and number is measured in a Coulter counter by passing a 0.5 cm^3 sample of cell suspension through a 100 uM diameter aperture. A current path exists between two electrodes placed either side of this aperture. As a cell passes through the aperture it displaces a volume of electrolyte equal to its own volume and increases the resistance between the two electrodes. The consequence of the increase in resistance is the generation of a voltage pulse proportional to the increase in resistance and hence volume. A threshold circuit discards small voltage pulses caused by cell debris. The Coulter counter is calibrated with latex spheres of known size and the aperture size, aperture current, threshold voltages and window width are optimised for counting

particles in the range of 10 - 25 uM diameter (Boardman et al, 1974). The Coulter counter output is fed into a pulse height analyser (Coulter Channelyser). The channelyser counts the voltage pulses from the Coulter counter and assigns them on the basis of voltage to one of a hundred discrete channels. In this way a frequency : voltage (volume) distribution curve for any cell sample can be produced. Cell number can simply be calculated by integrating the area under the curve and applying the appropriate correction factor for dilution and for calibrating voltage into volume. This calculation, and those for mean cell volume and mean cell water content, were usually performed upon an Olivetti P6060 mini-computer. For these calculations it was assumed that trypsinisation although altering cell shape had no effect upon cell volume, and that the cell water to volume ratio was 0.8 (Burrows & Lamb, 1962; Boardman et al, 1974; Aiton et al, 1981b).

(iii) Preparation of MDCK Cell Monolayers upon Permeable Filter Supports

For transport studies akin to those performed upon in vitro frog skin and toad urinary bladder, MDCK cells were grown upon a permeable support. MDCK cells have been grown successfully on three different substrates; untreated millipore filters, 5 uM pore diameter (Misfeldt et al, 1976); collagen coated nylon nets (Cereijido et al, 1978) and collagen coated nucleopore filters, 5 uM diameter (Rabito et al, 1978). Since similar results were obtained with all three preparations it was decided to use the simplest solution - untreated millipore filters. Two sizes of filters were used - 25mm diameter and 47mm diameter. A smaller pore diameter - 0.22uM was used to provide more support for the cells.

To prepare the filters for use they were boiled in distilled water for ten minutes to remove the wetting agents added during manufacture. The

25mm diameter filters were mounted into Swinnex millipore filter holders (Millipore Corp.) and sterilised in an autoclave. The 47mm diameter filters were autoclaved mounted between two sheets of glass. To seed the filters with cells two different techniques were employed: Filters mounted in Swinnex holders were seeded by introducing a cell suspension into the swinnex - the area between the filter and the top of the swinnex acting as a reservoir - capacity $\sim 2cm^3$. The holders plus cell suspension were incubated at 37°C in a 95%/5% air/CO_2 atmosphere during which time the cells come out of suspension and attach to the filter substrate. The filters were then removed from the holders and placed cell surface uppermost in individual 5cm diameter plastic petri-dishes containing 5 cm^3 complete growth media supplemented with 1 I.U. cm^{-3} insulin as a growth stimulant (Misfeldt et al, 1976). 47mm diameter millipore filters were loaded by placing them individually in 5cm diameter petri-dishes and adding 5 cm^3 of a 2 x 10^6 cells.cm^{-3} cell suspension. The dishes were then incubated at 37°C in a 95%/5% air/CO_2 atmosphere for one hour. The filters with cells attached were then transferred to 9cm diameter plates containing 20 cm^3 complete growth media supplemented with insulin. After 3-4 days incubation at 37°C in a 95%/5% air/CO_2 atmosphere the cells had formed confluent monolayers and were then used for experimental purposes. Monolayer confluence was ascertained by microscopic analysis of randomly selected monolayers and more routinely on the basis of the observed transepithelial resistance (Simmons, 1981).

(iv) Preparation of MDCK Cell Subconfluent Monolayers upon Plastic Petri-dishes.

In addition to monolayers grown upon permeable filter supports, transport

studies were performed upon subconfluent cell monolayers grown upon plastic petri-dishes (3 and 5 cm diameter). The initial seeding densities for these plates 0.9×10^5 cells (3cm) and 2.5×10^5 cells (5cm) in either 2 cm^3 or 3 cm^3 complete growth media. For experimental purposes the plates were used after 3-4 days of growth when they were still subconfluent (final cell densities in the order of $1\text{-}3 \times 10^6$ cells.$plate^{-1}$). Subconfluent cell monolayers were used in a number of experiments because they have less extracellular space than is found in confluent monolayers. For measurement of non-equilibrium ^{86}Rb influx and efflux rates this is a distinct advantage (see Chapter 5).

(B) Electrophysiological Studies.

(i) Chamber Design.

The majority of the experiments described in this thesis were made using 2.5 cm diameter cell monolayers mounted between two lucite Ussing-type half chambers. The chambers were arranged in blocks of six and were thermostated at 37°C by means of a forced water circulation through conduits cut into the lucite block. Each chamber had a 0.75 cm radius window so that the exposed tissue area was 1.76 cm^2. The volume of each half chamber was 7 cm^3. Despite care taken in mounting MDCK cell monolayers in these chambers, monolayers were subject to some degree of edge damage (Helman & Miller, 1973), due to the compression of the cell layer between the chamber edges (Barker & Simmons, 1981; Simmons, 1981). The effect of edge damage is to introduce an additional paracellular 'shunt' pathway across an epithelium. Although this additional 'shunt' is of little quantitative significance in low resistance epithelia, due to the large contribution of the paracellular route to total trans-

epithelial conductance under normal circumstances, it assumes importance in 'tight' epithelia which normally exhibit high transepithelial resistance/low paracellular 'shunt' profiles (Helman & Miller, 1973). To overcome this problem a new set of chambers (fig 2.1) was designed to minimise the effect of edge damage upon high resistance monolayers. This was achieved in two major ways:

1. The edge-to-surface area ratio was improved by using larger monolayers and chambers (exposed monolayer area was increased from 1.76 cm^2 to 9.2 cm^2). In this way the contribution of the damaged edge to total monolayer resistance was limited (Dobson & Kidder, 1968).
2. An improved clamping technique was employed whereby isolation of the damaged area from the area from which measurements were being made by incorporating a viscous oil seal within the damaged area.

Figs 2.2 and 2.3 show histograms of the magnitude of potential and electrical resistance generated by MDCK cell monolayers grown upon 2.5 cm diameter filter supports (fig 2.2) and those grown upon 4.7 cm diameter filters and mounted into the improved chambers. The mean potential and resistance generated by cell monolayers mounted without edge damage: 5.9 ± 0.5 mV and 7.9 ± 0.6 k $\Omega.cm^2$, n = 77, compared to 1.2 ± 0.1 mV and 3.5 ± 0.3 k $\Omega.cm^2$, n = 123, clearly illustrates that edge damage caused by crushing introduces considerable error into the estimation of the electrical properties of high resistance epithelia. Fig 2.4 shows that for monolayers mounted without edge damage, a linear relationship between potential and resistance still exists (see Higgins et al, 1975 and Simmons, 1981a). However an inverse relationship between potential and resistance is observed during hormone stimulation of transport (fig 2.5). Taken together these results imply that even

in monolayers mounted without edge damage a significant paracellular leak pathway still exists, a possible candidate being mitotic cells.

(ii) Electrode Design

Transepithelial potential, resistance and short circuit current (SCC) were measured using an automatic voltage clamp device originally designed to measure these parameters in rabbit isolated small intestine (Simmons & Naftalin, 1976; Naftalin & Simmons, 1979).

The potential sensing electrodes were reversible calomel half-cells ($Hg/HgCl_2/KCl$). Electrode pairs were matched and electrodes were discarded if there was a standing potential of more than 1 mV between any pair. A compensation circuit consisting of a battery and variable potentiometer was provided for in the design of the apparatus to nullify any residual potentials in the circuit due to asymmetries in the electrodes or salt bridges. All potentials are expressed relative to the basal surface of the cell monolayer.

The current passing electrodes were large area silver/silver chloride half-cells (Ag/AgCl/KCl), constructed by coating a silver wire coil with molten silver chloride.

Both sets of electrodes were connected to each Ussing chamber via small diameter ($\sim$1mm internal diameter) low resistance saturated KCl-agar salt bridges. The use of small diameter KCl salt bridges minimised errors due to liquid junction potentials, important in studies involving asymmetric ion replacements, and also prevented contamination of the bathing solutions with silver ions (Klyce & Marshall, 1982).

The optimum electrode/chamber geometry was assured by drilling guide holes for the salt bridges into the chambers so that:

1. The tip of the KCl salt bridge from the potential sensing electrode

was held within 2 mm of the monolayer surface and close to its centre. 2. The tip of the KCl salt bridge from the current passing electrode was held in line with the monolayer's centre at the furthest point from the monolayer. This arrangement ensured a uniform current density across the monolayer during current passage.

(iii) Experimental Solutions.

All experiments were carried out, unless otherwise stated, in a modified Krebs' solution of the following composition: 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 12 mM HCL, 14 mM Tris base, 10 mM glucose and 2% (vol/vol) donor horse serum. Experiments were carried out in Krebs' solution in preference to growth media (EMEM) to avoid the added complications of using a bicarbonate buffer such as the need to gas the media with 5%/95% CO_2/air. The alternative was to use growth media with HEPES as the buffering agent. However in our hands HEPES appears to be cytotoxic to MDCK cells. Table 2.2 validates the use of Krebs' solution as a suitable alternative to growth media showing that over a two hour period there was no significant decrease in cell monolayer viability. The Krebs' solution was not gassed for two main reasons: (1) MDCK cells have a primarily anaerobic metabolism (Misfeldt et al, 1976) and (2) to avoid "sparge" damage to the monolayer. Also, with the inclusion of serum in the buffer, foaming would become a problem.

A sodium-free solution was prepared by replacing NaCl with either choline chloride, LiCl or Tris chloride, NaH_2PO_4 by KH_2PO_4 and normal serum by serum which had been dialysed against distilled water (50 x volume) at 4°C for 24 hours. Solutions with varying Na^+ ion concentrations were prepared by mixing a Na^+-free solution (Na^+ replaced by

choline) with normal (137 mM) Krebs' in appropriate proportions. The Na^+ ion content of all Krebs' solutions were checked by flame photometry.

A chloride-free Krebs' solution was prepared in a similar manner. NaCl was replaced by either NaBr, NaSCN, NaI, $NaNO_3$ or Na isethionate, KCl by K_2SO_4, CaCl by $CaSO_4$ and HCl by H_2SO_4. Dialysed serum was used and isosmolarity was maintained by the addition of the appropriate amount of mannitol. Solutions with varying Cl^- concentrations were prepared in a similar manner to Na^+ solutions using a $NaNO_3$ Krebs' as the replacement.

(iv) Measurement of Transepithelial Resistance.

Transepithelial resistance was determined in two ways: First - under open circuit conditions resistance was determined by passing a small 2-5 uAmp hyperpolarising current pulse across the monolayer and measuring the magnitude of the voltage deflection. The second method was employed to determine resistance during flux measurements under short circuit conditions. In this situation the resistance was determined by Ohm's Law from the short circuit current and the open circuit transepithelial voltage generated when the clamp was released. In both cases voltage was measured after thirty seconds to allow the potential to reach a steady-state value.

(C) Net Ion Fluxes

(i) Na^+ Ion Flux

Transepithelial net Na^+ movements from apical to basal (J_{a-b}) and from basal to apical (J_{b-a}) bathing solutions were measured simultaneously upon the same monolayers voltage clamped to 0 mV transepithelial

potential, using ^{22}Na and ^{24}Na as tracers for Na^+. Flux measurements were initiated by the addition of 1 uCi.cm^{-3} of ^{24}Na and ^{22}Na to the apical and basal bathing solutions respectively. The volume of each half chamber was 7 cm^3 and the volume of isotope added never exceeded 0.1 cm^3. A 1 cm^3 sample was taken from each half chamber after twenty, fifty and eighty minutes incubation. Fluxes were averaged over the half-hour periods, the initial twenty minute period was to allow for tracer equilibration across the apical and basal cell membranes. The effect of drugs upon the bi-directional Na^+ fluxes were studied by adding the drug to the appropriate bathing solution at the start of the second half-hour flux period (fifty minutes). Short circuit current was recorded continuously over the flux periods. Trans-epithelial resistance was also determined at ten minute invervals throughout the flux periods (as described above). Flux was calculated from the appearance of radioactive tracer in the contra-lateral half chamber and the specific activity of the tracer added to the opposing half chamber and is expressed as u moles.cm^{-2}.h^{-1}. Tracer equilibration in the contra-lateral chamber was always less than 0.05% of the 'hot' side activity, so minimising any errors due to back-flux. ^{24}Na activity was determined by its γ -emissions in a Prias Autogamma counter. Correction for activity due to the presence of ^{22}Na in the sample was made after recounting the samples following the complete decay of ^{24}Na (10 half-lives). ^{22}Na activity was determined after ten ^{24}Na half lives from its β -emissions in a Packard liquid scintillation spectrophotometer (model 3255). 10 cm^3 of a scintillation cocktail (45% vol/vol toluene, 45% vol/vol Triton X-100 and 10% vol/vol Scintol-2) was added to each vial.

(ii) K^+ Ion Fluxes

Transepithelial bi-directional K^+ ion fluxes were measured in a similar manner as bi-directional Na^+ ion fluxes. ^{42}K was used to measure the apical to basal (J_{a-b}) unidirectional flux and ^{86}Rb was used to measure the reverse basal to apical (J_{b-a}) flux. The suitability of ^{86}Rb as a tracer for transepithelial K^+ ion movements was determined in a series of control experiments in which unidirectional fluxes were measured simultaneously by both ^{42}K and ^{86}Rb (fig 2.6). Over a wide range of K^+ fluxes ^{86}Rb can be seen to be an adequate tracer of K^+ movements. ^{42}K activity was measured by its γ -emissions in a Prias Autogamma counter. Correction for ^{86}Rb activity in a mixed sample was made by recounting all samples after ten half-lives. ^{86}Rb activity was counted after ^{42}K decay by its β -emissions in a Packard liquid scintillation counter (model 3255) in 10 cm^3 of scintillation cocktail.

(iii) Cl^- Ion Fluxes

Initially transepithelial Cl^- movements were measured by a one isotope technique. ^{36}Cl was used as the tracer. Uni-directional fluxes were determined upon adjacent monolayers or upon the same monolayer sequentially in a randomised order to avoid systematic error due to deterioration in monolayer condition during the thirty minute flux periods. In some cases transepithelial resistance fell after washing out the tracer from the 'hot' side and these monolayers were rejected if the resistance drop was more than 5% of the initial resistance. The effect of drugs upon net Cl^- flux was measured during the second flux period in a similar manner to that for Na^+ and K^+ fluxes. In later experiments, when isotope became available, the accuracy with which Cl^- fluxes were measured was improved by the introduction of a

simultaneous double label technique using ^{77}Br as the second tracer for Cl^- movements. These experiments were also performed in the improved chamber design. An additional feature of this chamber to those already mentioned (Section B(i)) was the increased surface area of cells to the volume of the chamber ratio. This leads to an improved accuracy in measuring small fluxes (tracer equilibration was still less than 0.5%). The suitability of ^{77}Br as a tracer was tested by measuring the same uni-directional flux simultaneously using both tracers (fig 2.7). A correction factor is applied to ^{77}Br flux measurements. ^{77}Br activity was measured in samples by its γ-emissions on a Prias Autogamma counter. ^{36}Cl activity was determined in a Packard liquid scintillation counter, in 10 cm^3 of scintillation cocktail.

(D) Rapid K^+ Flux Measurements

(i) K^+ Influx into Sub-confluent Monolayers of MDCK Cells.

The rate of K^+ uptake into MDCK cells was measured upon sub-confluent monolayers of MDCK cells grown upon plastic petri-dishes. The monolayers were used in a sub-confluent state to allow ease of access of experimental solutions to the basal aspects of the monolayers, and to reduce problems with extracellular space (see above). As was the case in the measurement of net transepithelial K^+ movements, ^{86}Rb was found to be a more convenient tracer than ^{42}K due to its longer half-life (18.7 days). The suitability of ^{86}Rb as a tracer for rapid K^+ influx was determined in control experiments where both ^{42}K and ^{86}Rb were used to simultaneously measure a wide range of K^+ ion influx rates (fig 2.8): ^{86}Rb influx did not differ significantly from a 1:1 ratio from the corresponding ^{42}K measured influx. K^+ influx determinations were made

at 37°C over a five minute flux period. A five minute flux ensured that all flux determinations were made within the linear portion of the ^{86}Rb uptake curve and so represent true initial rates of K^+ influx into the cells (fig 2.9). The procedure for measuring ^{86}Rb influx into sub-confluent monolayers of MDCK cells was as follows:
The growth media was removed from sub-confluent monolayers of MDCK cells and the monolayers rinsed 4x with Krebs' solution (20 seconds). For monolayers on which fluxes were to be determined in the presence of ouabain this pre-incubation wash contained ouabain (1×10^{-4}M). Flux was initiated by the addition of 2 cm^3 of the appropriate experimental solution (containing 0.2 $uCi.cm^{-3}$ ^{86}Rb) to each plate. At the end of the influx period the cell layer was rinsed 4x with ice-cold Krebs' solution, a procedure that removed all extracellular isotope. This was confirmed in separate control experiments using ^{14}C-inulin as an extracellular space marker. The cell layer was then treated with 1 cm^3 of a trypsin/EDTA solution (0.25% vol/vol 2mM in a Ca^{2+}/Mg^{2+} free salt solution). The enzymic action of trypsin was then neutralised with the addition of 2 cm^3 of Krebs' solution - any remaining cell clumps were dispersed by repeated syringing of the suspension through a wide bore needle. The cell number and cell volume of a 1 cm^3 aliquot from each plate was determined upon a Coulter counter with channelyser (see above). The ^{86}Rb activity of a second 1 cm^3 aliquot from each plate was determined by the Cerenkov effect in a Packard liquid scintillation counter. Each sample was diluted in 10 cm^3 of distilled water. Samples were corrected for colour quench by counting standards plus and minus 1 cm^3 of a trypsin/Krebs solution (1 cm^3/2 cm^3). Experimental blanks were taken to be the counts

recovered from cell-free petri-dishes taken through the experimental procedure outlined above.

(ii) Measurement of K^+ Influx Across the Apical and Basal Cell Membranes

The contribution of flux across either cell membrane to the total cellular K^+ ion influx was determined upon confluent monolayers of MDCK cells grown upon permeable filter supports and mounted in Ussing chambers (1.76 cm^2 exposed monolayer area). Influx measurements were initiated by adding 7 cm^3 of Krebs' solution containing 0.2 $uCi.cm^{-3}$ ^{86}Rb to the appropriate half chamber. Solutions were mixed by small stir bars in each half chamber. After a five minute influx period the radioactive solution was removed by suction and the monolayer rinsed 4x with ice-cold Krebs. The filters were then placed in scintillation vials and the isotope extracted into 10 cm^3 distilled water. ^{86}Rb activity of samples was determined by the Cerenkov effect in a Packard liquid scintillation counter. An estimate of cell number and cell volume was made by trypsinising cell monolayers from several filters of the same batch as used to measure K^+ influx and counting on a Coulter counter with channelyser attachment (see above).

(iii) Measurement of K^+ Efflux Rates from Sub-confluent Monolayers of MDCK Cells.

The rate of K^+ ion efflux from sub-confluent monolayers of MDCK cells was investigated using a technique described by Aiton and Lamb (1980) for HeLa cells. Briefly, MDCK cells were loaded for three hours with ^{86}Rb (0.5 $uCi.cm^{-3}$ growth media) at 37°C. After this time ^{86}Rb content of the cells is in equilibrium and represents 92% of the cellular K^+, as measured by flame photometry (Aiton et al, 1981b). The cell layers were then rinsed briefly (4x) with Krebs' solution (twenty seconds) at

37°C. ^{86}Rb efflux was then measured by the successive addition and collection of 2 cm^3 aliquots of isotope free Krebs' solution at two minute intervals. At the end of the efflux period the cell monolayers were rinsed briefly (4x) with ice-cold Krebs' solution (twenty seconds) to remove any extracellular isotope. The ^{86}Rb activity remaining in the cells at the end of the efflux period was estimated from an aliquot of cell suspension prepared by trypsinisation as described above. The suitability of ^{86}Rb as a tracer for K^+ efflux, including drug stimulated efflux, was determined in separate control experiments in which ^{42}K and ^{86}Rb were used to measure the same efflux simultaneously (fig 2.10). ^{86}Rb activity was estimated from the Cerenkov effect. A colour quench correction was made for the ^{86}Rb content remaining in the cells (see above). Cell-free plates were used to run blanks.

(iv) K^+ Efflux from Confluent MDCK Cell Monolayers.

The contribution of flux across either cell membrane to the total cellular K^+ efflux rate was determined in a similar manner to that used to determine K^+ influx. MDCK monolayers on permeable filter supports were pre-loaded with ^{86}Rb (0.5 $uCi.cm^{-3}$ growth media) for three hours. The cell monolayers were then rinsed briefly in Krebs' solution (twenty seconds) at 37°C and mounted into Ussing chambers (1.76 cm^2 exposed monolayer area). Flux was initiated by adding 5 cm^3 of Krebs' solution to the apical and basal half chambers simultaneously. Efflux across both membranes was measured by the successive collection (by suction) and addition of 5 cm^3 aliquots of Krebs' at two minute intervals. At the end of the experiment cell monolayers were rinsed briefly with ice-cold Krebs' and the remaining ^{86}Rb activity extracted in 10 cm^3 of distilled water. The effect of hormones and drugs upon fractional K^+

efflux was investigated either by adding the drug at t=0 minutes or by adding it at the start of the experiment.

(v) Calculation of Fractional ^{86}Rb Effluxes from MDCK Cells.

Fractional ^{86}Rb efflux rates were calculated from the following equations:

$$F_p = (C_B)_p \,/\, (C_t + (\sum_{i=p}^{pf}(C_A)_i + \sum_{i=p}^{pf}(C_B)_i - \tfrac{1}{2}\,((C_A)_p + (C_B)_p)))$$

and for apical K^+ efflux:

$$F_p = (C_A)_p \,/\, (C_t + (\sum_{i=p}^{pf}(C_A)_i + \sum_{i=p}^{pf}(C_B)_i - \tfrac{1}{2}\,((C_A)_p + (C_B)_p)))$$

where for each time period (p): (C_A) and (C_B) are the ^{86}Rb activities appearing in the apical and basal bathing solutions respectively. C_t is the ^{86}Rb activity remaining in the cells at the end of the flux period.

$\sum_{i=p}^{pf}(C_A)_i$ and $\sum_{i=p}^{pf}(C_B)_i$ are the activities of ^{86}Rb appearing in the apical or basal bathing solution respectively, from period p to the final period pf.

For fractional effluxes from sub-confluent plates the following equation applies:

$$F_p = C_{mp} \,/\, (C_t + \sum_{i=p}^{pf}(C_p)_i - \tfrac{1}{2}C_{mp})$$

where for each time point (p): C_{mp} is the total ^{86}Rb activity appearing

in the media during time period p. C_t is the total ^{86}Rb activity remaining in the cells at the end of the experiment.

$\sum_{i=p}^{pf} (C_p)_i$ is the ^{86}Rb activity appearing in the media from period p to the final period pf.

(E) Measurement of Intracellular Ion Contents.

The effect of various experimental procedures upon net ion contents of sub-confluent cell monolayers upon plastic petri-dishes was determined in the following way: Cells were incubated in the appropriate experimental solution then washed (4x) with ice-cold isotonic sorbitol solution (twenty seconds). This washing procedure, identical to that used in influx experiments, effectively reduces ion concentrations in the extracellular space. The intracellular ions were extracted from the cells by a two hour incubation in 2 cm^3 of distilled water at room temperature. Ion contents were then measured by flame photometry using a 100 $uEq.l^{-1}$ Na^+ and K^+ standard. The cell number and cell volume of identical cell monolayers were determined, after trypsinisation, on a Coulter counter with channelyser attachment. Ion contents are expressed as $mMoles.l^{-1}$ cell water.

(F) Statistical Analysis.

Statistical variance of grouped data, except where stated, is routinely expressed as the standard error of the mean (S.E.M). Statistical comparisons were made using Student's t-test (unpaired means solution). Significance levels were obtained by comparing calculated values of t

with the tabulated distribution of t (Snedecor & Cochran, 1968). Least square regression analysis was performed on an Olivetti P6060 mini-computer using an Olivetti statistical package. Log dose response curves were analysed using a Probit analysis performed on an Olivetti P6060.

TABLE 2.1

The main constituents of Eagle's Minimum Essential Media with Earles salts. Further details are available in the Flow Laboratories Tissue culture manual.

amino acids	$mg.l^{-1}$
L-arginine HCl	126.4
L-cysteine	28.4
L-glutamine	292.3
L-histidine	41.9
L-isoleucine	52.5
L-leucine	52.5
L-lysine	73.0
L-methionine	14.9
L-phenylalanine	33.0
L-threonine	47.6
L-tryptophan	10.2
L-tyrosine	36.2
L-valine	46.9

Inorganic salts	$mg.l^{-1}$
$CaCl_2.2H_2O$	264.9
KCl	400.0
KH_2PO_4	60.0
$Mg.SO_4.7H_2O$	200.0
NaCl	6800.0
$NaHCO_3$	2000.0
$NaH_2PO_4.2H_2O$	158.3

Other ingredients	
glucose	$1000 mg.l^{-1}$
foetal bovine serum	5% (v/v)
donor horse serum	5% (v/v)
kanamycin	$100 ug.cm^{-3}$

TABLE 2.2

Validation of the use of a Krebs' solution as the experimental buffer. The results show that over a two hour incubation period there was no significant decline in either transepithelial electrical resistance or in the mean open circuit potential the monolayers maintained. The results were obtained from large area (9.62 cm^2) monolayers mounted without appreciable edge damage in Ussing type chambers. The results are expressed as the mean $\pm$ SEM of 6 separate determinations.

Time (mins)	potential (mV)	resistance ($k\Omega.cm^2$)
2	6.2 ± 0.4	9.2 ± 0.8
5	5.8 ± 0.3	8.6 ± 1.2
10	5.6 ± 0.6	8.7 ± 0.5
30	5.7 ± 0.6	8.5 ± 0.7
60	5.4 ± 0.4	8.6 ± 0.6
90	5.3 ± 0.2	8.5 ± 0.4
120	5.2 ± 0.3	8.5 ± 0.5

Fig 2.1 Schematic diagram of the improved Ussing chamber design.

A cross section through the new chambers shows: that an improved surface area to chamber volume was achieved by using conical half chambers (a). The cell monolayer, upon a permeable filter support (b) is mounted between two such half chambers. Mechanical mounting is achieved by clamping the filter support between two rubber 'O' rings (c) mounted upon either half chamber. To minimise the effects of crush damageupon electrical measurements an oil seal (d) is placed inside the mounting points. To ensure optimum electrode placement KCl-agar elctrodes are inserted through guide holes (e) drilled at the appropriate points. Access to the half chamber is through a sampling port on the upper surface of the chamber (f).

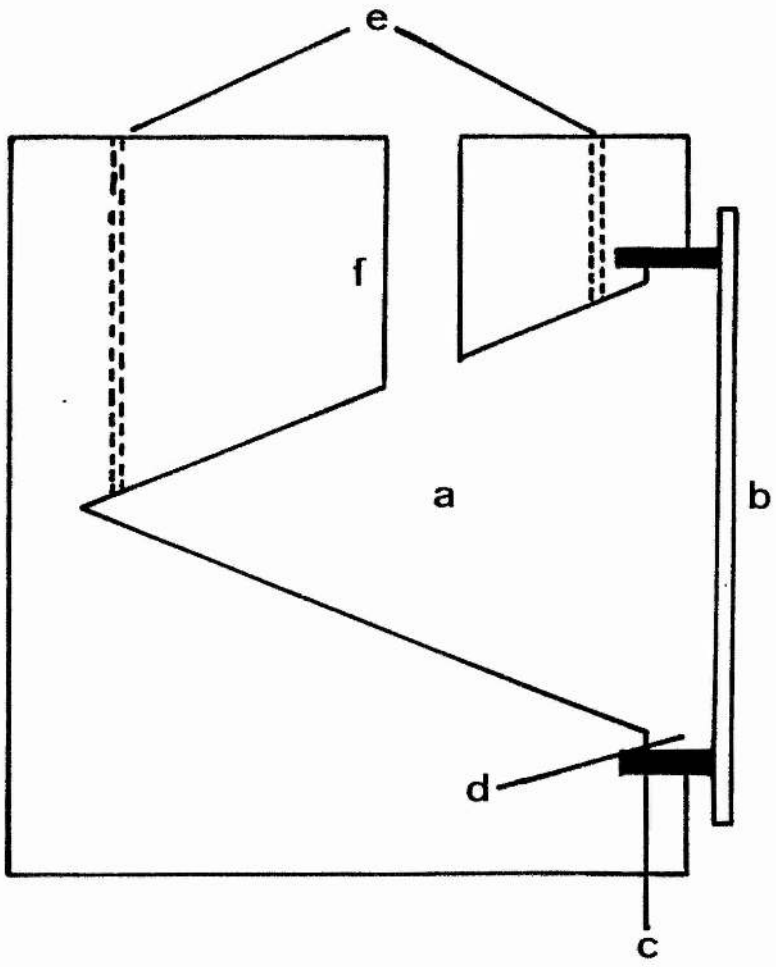
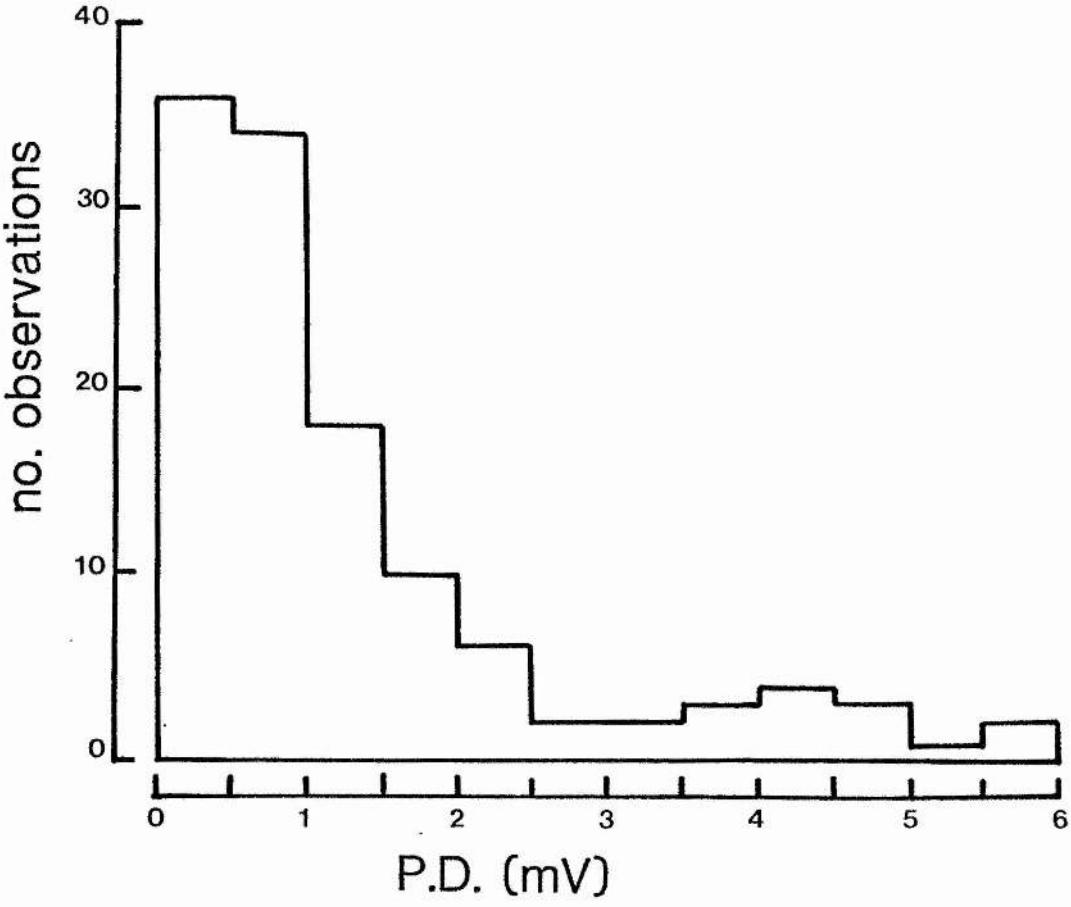

e
f
a
b
d
c

Fig 2.2a Histogram of the open circuit spontaneous potential generated by MDCK cell monolayers.

Abscissa: number of observations. Ordinate: potential (mV), 0.5 mV band width. The open circuit potential of epithelial monolayers of MDCK cells (strain I) was measured 10 minutes after mounting in Ussing chambers (1.76 cm^2 monolayer area). The mean open circuit potential was 1.21 $\pm$ 0.11 mV basal-lateral cell surface positive.

Fig 2.2b Histogram of the transepithelial electrical resistance maintained by strain I MDCK cells.

Abscissa: number of observations. Ordinate: transepithelial resistance ($k\Omega.cm^2$), band width was 1 $k\Omega.cm^2$. Transepithelial electrical resistance was measured 10 minutes after mounting the cell monolayers in Ussing chambers. The mean transepithelial resistance was 3.54 $\pm$ 0.33 $k\Omega.cm^2$.


40
30
20
10
0
no. observations
0
1
2
3
4
5
6
P.D. (mV)

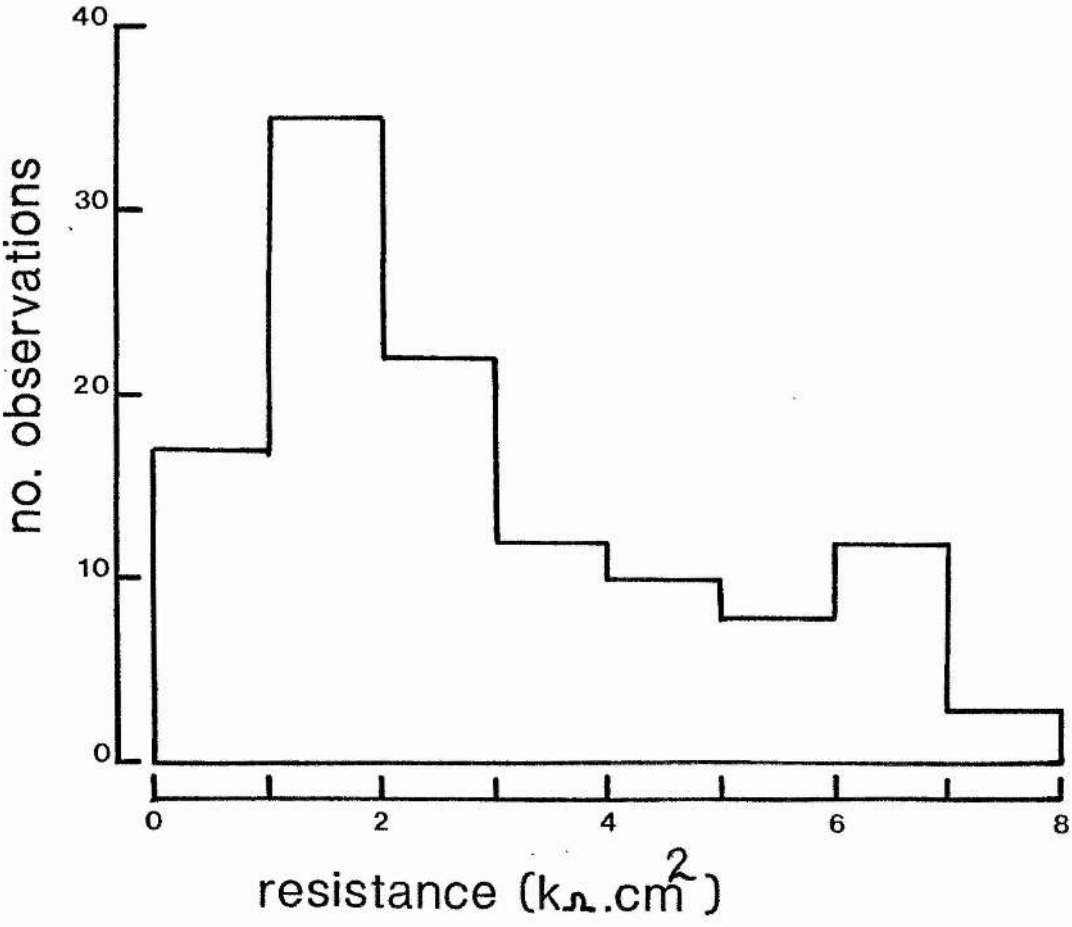

40
30
20
10
0
no. observations
0
2
4
6
8
resistance (kΩ.cm²)

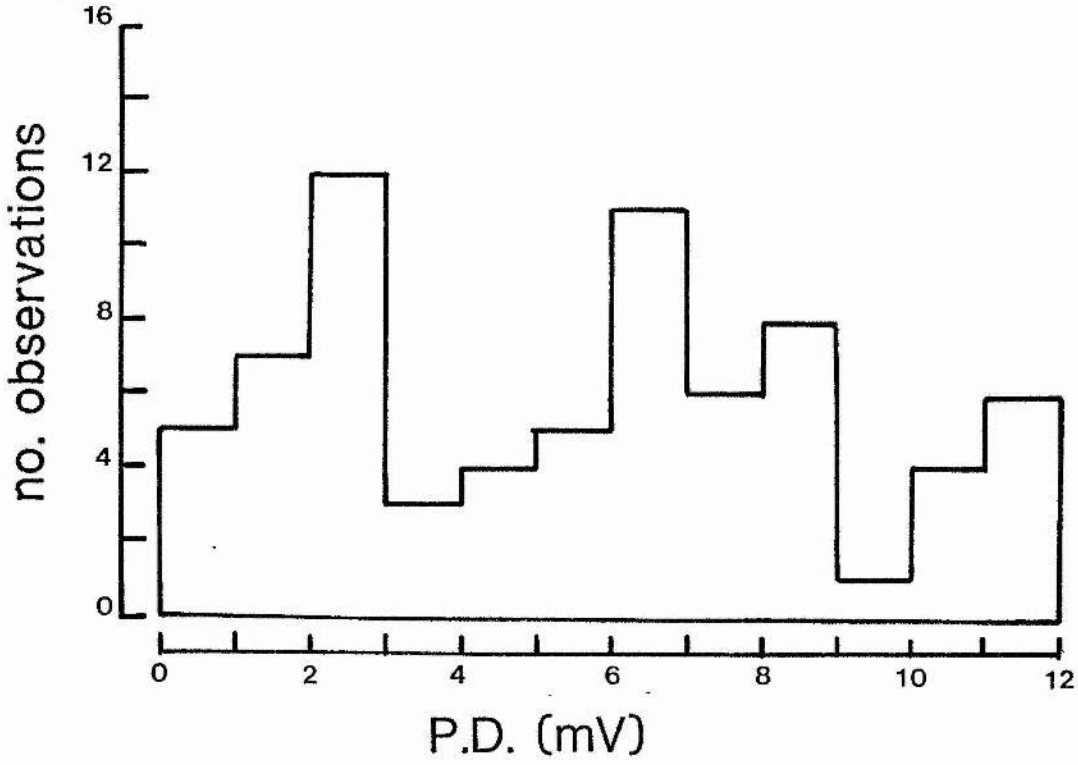
Fig 2.3a Histogram of spontaneous open circuit potential generated by MDCK cell monolayers, mounted free from edge damage.

Abscissa: number of observations. Ordinate: open circuit potential (mV), class width 1mV.

The mean open circuit potential was recorded 10 minutes after mounting the cell monolayers (9.62 cm^2 area) in the chambers described in fig 2.1. The mean open circuit potential was 5.9 ± 0.5 mV basal-lateral surface positive.

Fig 2.3b Histogram of the steady state transepithelial electrical resistance in MDCK cell monolayers mounted free from edge damage.

Abscissa: number of observations. Ordinate: transepithelial resistance ($k\Omega.cm^2$) the band width was 2 $k\Omega.cm^2$. The transepithelial resistance was determined 10 minutes after mounting the monolayers in Ussing chambers. The mean transepithelial resistance was 7.9 ± 0.6 $k\Omega.cm^2$.


no. observations
16
12
8
4
0
0
2
4
6
8
10
12
P.D. (mV)

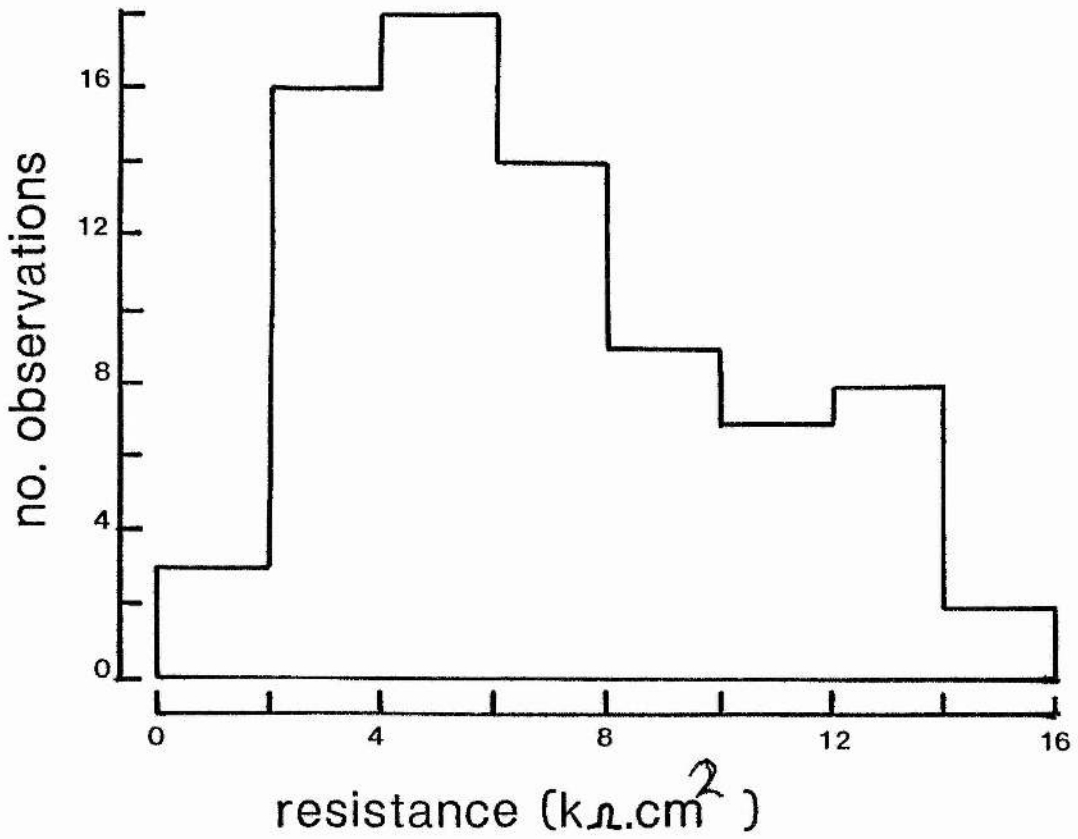

no. observations
16
12
8
4
0
0
4
8
12
16
resistance (kΩ.cm²)

Fig 2.4 Relationship between open circuit potential and transepithelial resistance in MDCK cell monolayers mounted free from edge damage. Abscissa: open circuit potential difference (mV). Ordinate: transepithelial electrical resistance ($k\Omega.cm^2$). This figure shows a scatter plot of the relationship between potential and resistance in large area (9.62 cm^2) MDCK cell monolayers mounted without edge damage. Potential and resistance were measured 10 minutes after mounting the monolayers in Ussing chambers. The solid line represents the least-squares regression line and obeys the equation:

$$y = 0.32(x) + 3.45$$

Both the slope of the line and the correlation coefficient (0.42) are significant ($P < 0.01$)

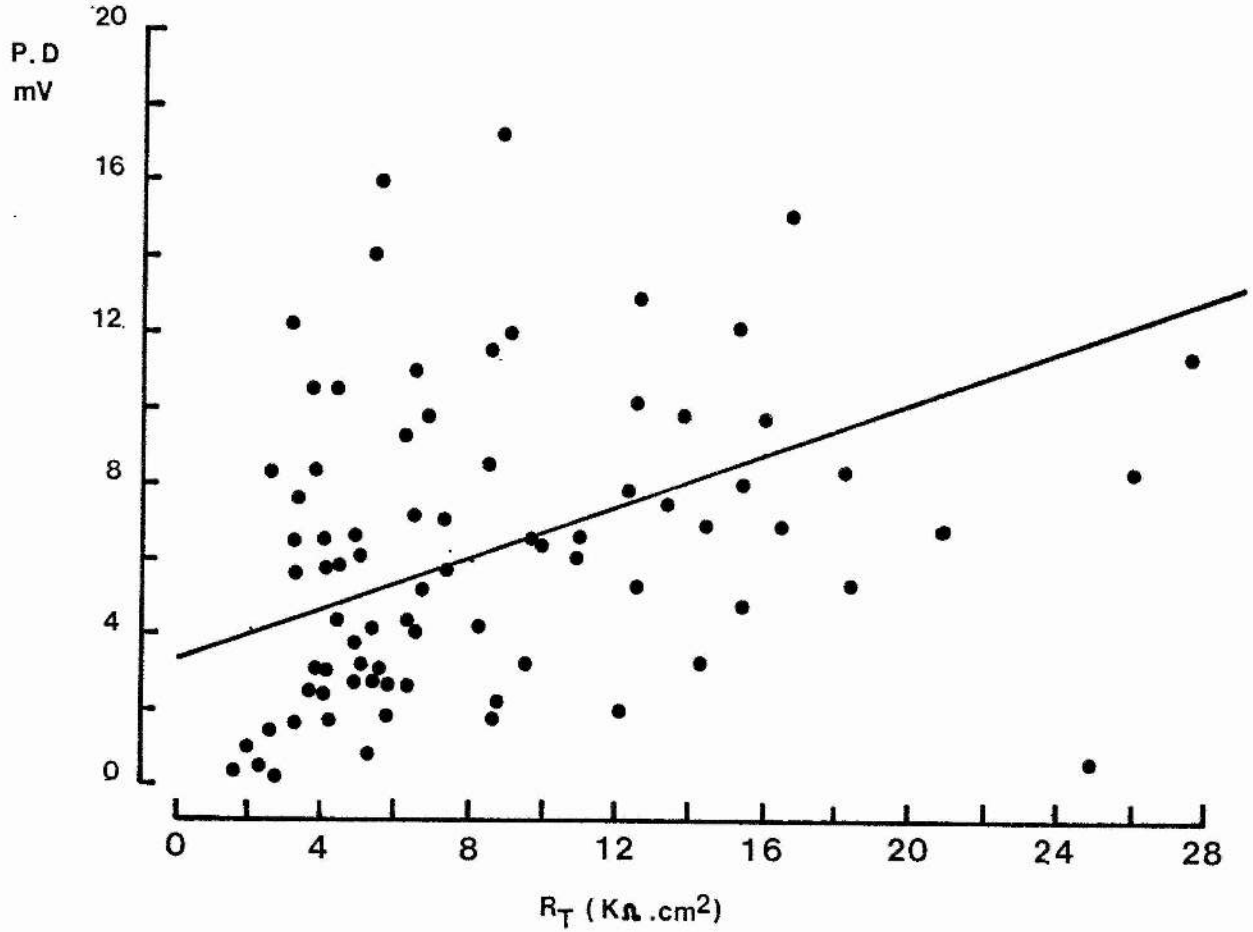

P.D
mV
20
16
12
8
4
0
0
4
8
12
16
20
24
28
R_T (KΩ.cm^2)

Fig 2.5 Inverse relationship between open circuit potential and transepithelial resistance during adrenaline stimulation.

Abscissa: open circuit potential (mV). Ordinate: transepithelial resistance ($k\Omega.cm^2$). The open circuit potential and transepithelial electrical resistance were recorded at various times after addition of 2 uM adrenaline to the basal-lateral bathing solution. The solid line represents the least-squares regression line and obeys the equation:

$$y = -2.65(x) + 29.1$$

Both the slope of the line and the correlation coefficient (0.66) are significant ($P < 0.01$, $n=30$).

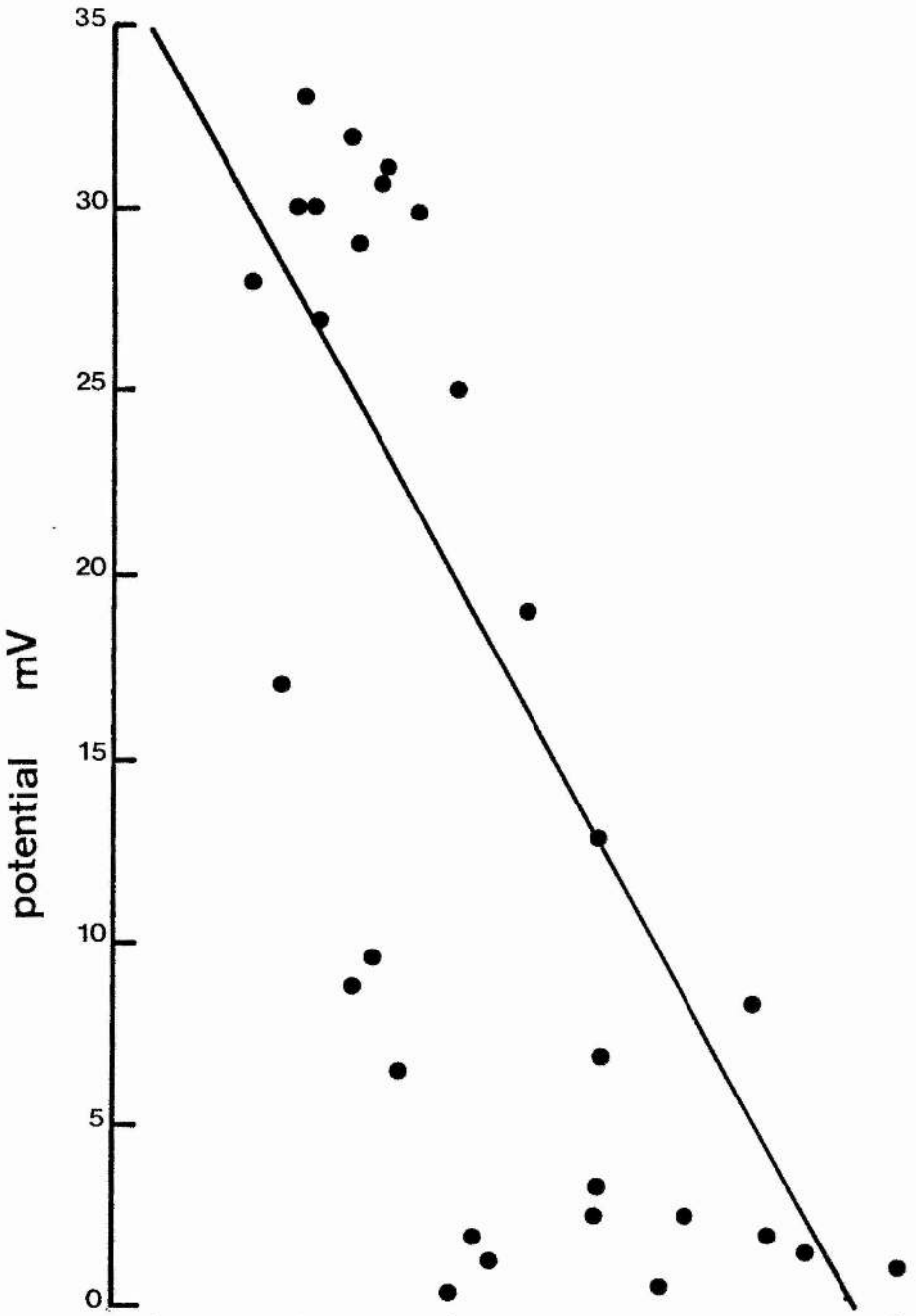

35
30
25
20
15
10
5
0
potential mV
0
2
4
6
8
10
resistance (kΩ.cm)

Fig 2.6 Validation of the use of ^{86}Rb as an adequate tracer for transepithelial K^+ movements.

Abscissa: ^{42}K flux ($nM.cm^{-2}.hr^{-1}$). Ordinate: ^{86}Rb flux ($nM.cm^{-2}.hr^{-1}$). The suitability of ^{86}Rb as a tracer for unidirectional K^+ fluxes was tested by using both ^{86}Rb and ^{42}K, simultaneously, to measure the same unidirectional K^+ flux. Fluxes were measured over two half-hour flux periods. In the first period ^{86}Rb and ^{42}K fluxes were measured under control conditions, in the second flux period ^{86}Rb and ^{42}K fluxes were measured in the presence of 2 uM adrenaline. The solid line represents the least-squares regression line and obeys the equation:

$$y = 1.0(x) - 1.6$$

Both the slope of the regression line and the correlation coefficient (0.992) were highly significant ($P < 0.001$). (●) control fluxes, (▲) flux plus 2 uM adrenaline.

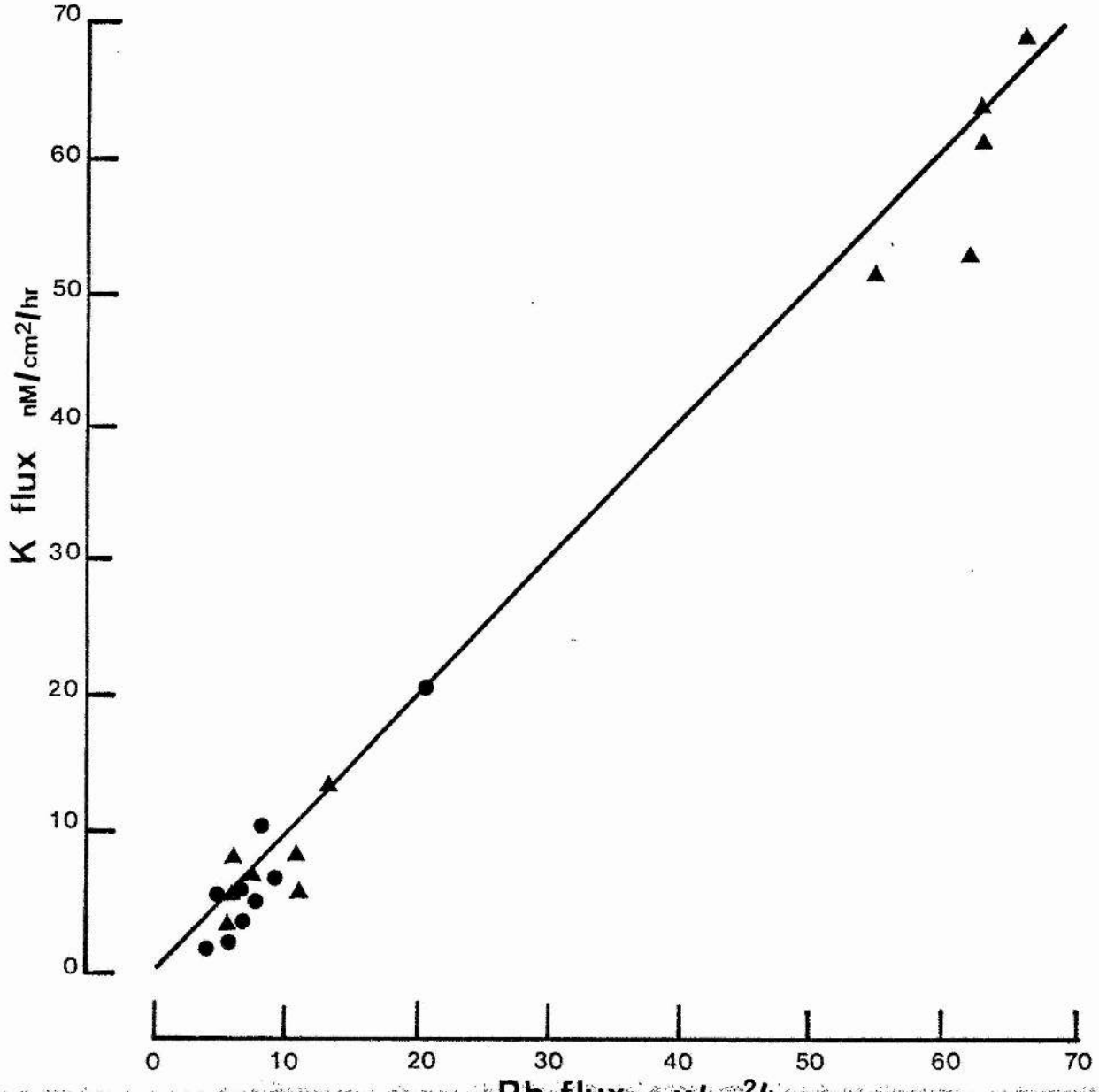

70
60
50
40
30
20
10
0
K flux nM/cm2/hr
0
10
20
30
40
50
60
70

Fig 2.7 Validation of the use of ^{77}Br as an adequate tracer for Cl^- movements.

Abscissa: ^{77}Br flux ($uM.cm^{-2}.hr^{-1}$). Ordinate: ^{36}Cl flux ($uM.cm^{-2}.hr^{-1}$). The suitability of ^{77}Br as a tracer for Cl^- movements was tested by measuring the same unidirectional Cl^- flux simultaneously with both ^{36}Cl and ^{77}Br as tracers. Flux was measured over two half-hour flux periods under control (●) and adrenaline (2 uM) stimulated (▲) conditions. The solid line represents the least-squares regression line and obeys the equation:

$$y = 1.48(x) - 0.06$$

Both the slope of the regression line and the correlation coefficient (0.980) were highly significant ($P<0.001$).

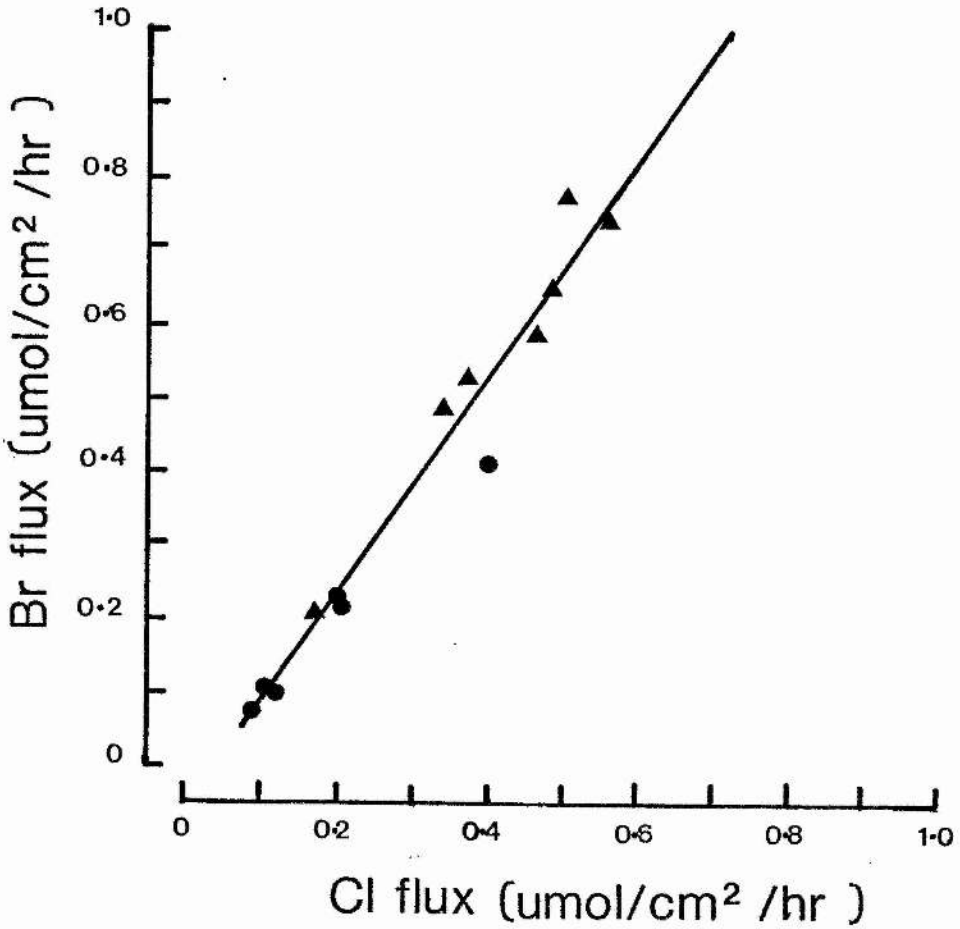

1·0
0·8
0·6
0·4
0·2
0
Br flux (umol/cm² /hr)
0
0·2
0·4
0·6
0·8
1·0
Cl flux (umol/cm² /hr)

Fig 2.8 Validation of the use of ^{86}Rb as an adequate tracer of K^+ influx into MDCK cells.

Abscissa: ^{86}Rb influx (mM/liter cell water/min). Ordinate: ^{42}K influx (mM/liter cell water/min). The suitability of ^{86}Rb as an adequate tracer for K^+ movements was tested by measuring K^+ influx into sub-confluent plates using both ^{86}Rb and ^{42}K as radioactive tracers. Influx was measured over a 5 minute flux period under control (●) conditions, and in the presence of 1mM ouabain (▲) or 0.1mM furosemide (■). The solid line represents the least-squares regression line for this data and was fitted by the equation:

$$y = 1.0(x) - 0.04$$

Both the slope of this line and the correlation coefficient (0.986) were highly significant. ($P<0.001$).

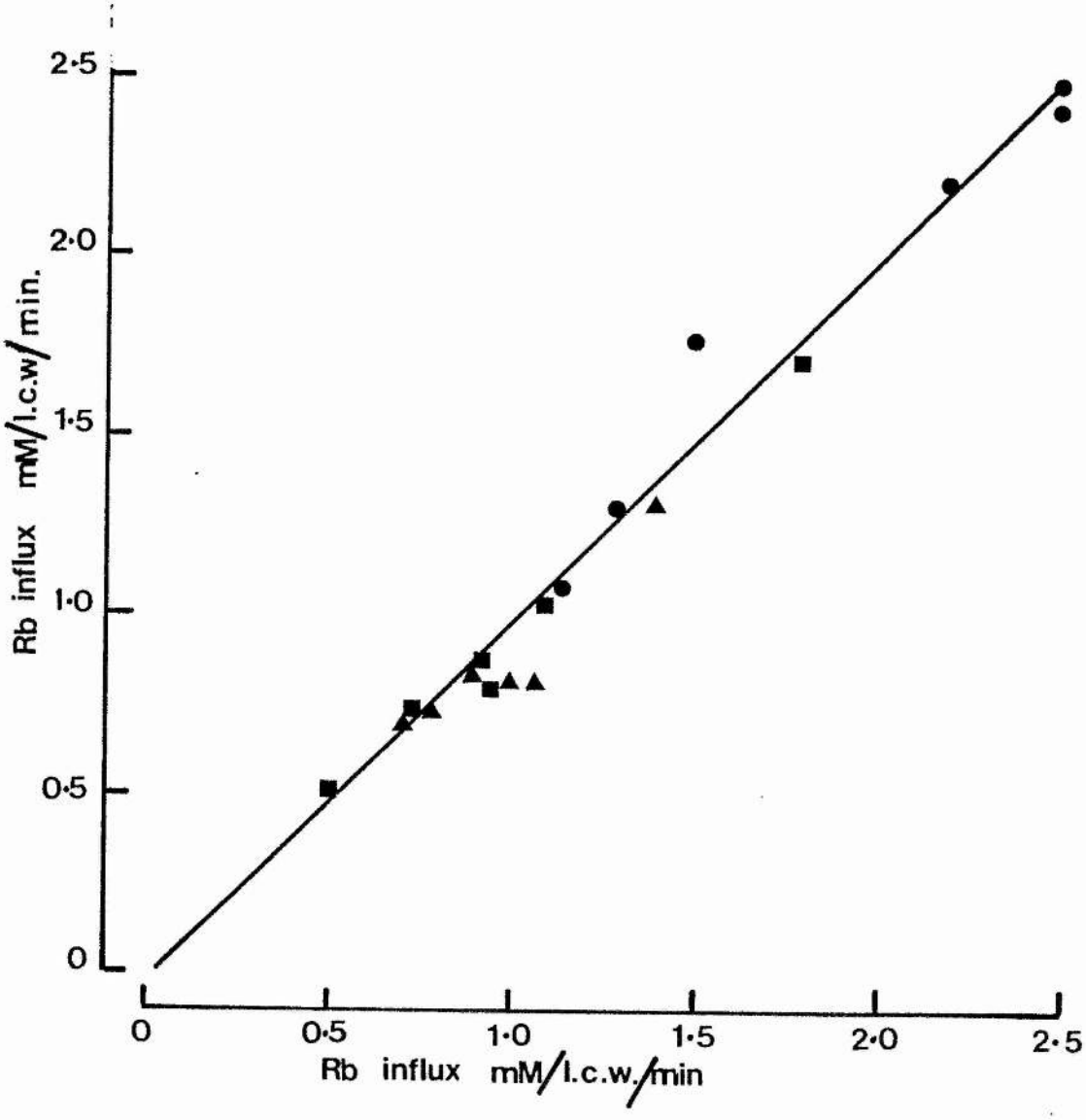

2·5
2·0
1·5
1·0
0·5
0
Rb influx mM/l.c.w/min.
0
0·5
1·0
1·5
2·0
2·5
Rb influx mM/l.c.w./min

Fig 2.9 ^{86}Rb uptake curve into subconfluent monolayers of MDCK cells.

Abscissa: ^{86}Rb uptake expressed as a fraction of total uptake at t = 3 hours. Ordinate: time (hours). ^{86}Rb uptake into subconfluent cell monolayers was measured at various times between 5 minutes and 3 hours. The resultant ^{86}Rb uptake curve shows that ^{86}Rb influx is linear for the first hour of uptake. To ensure measurements of ^{86}Rb influx were made in this linear portion of the curve ^{86}Rb influx was measured for either 5 minutes or 10 minutes. Each datum is the mean $\pm$ SEM of 3 separate determinations.

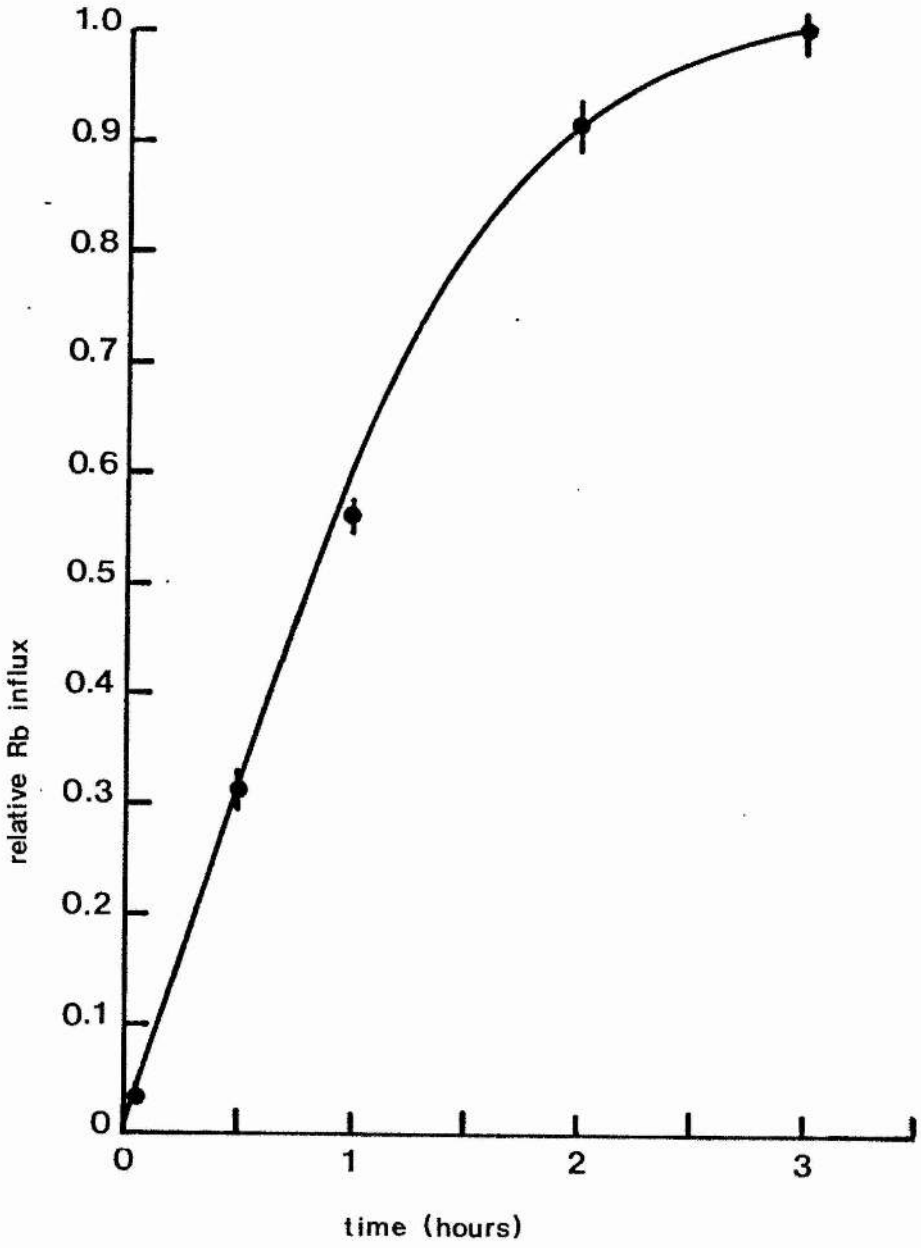
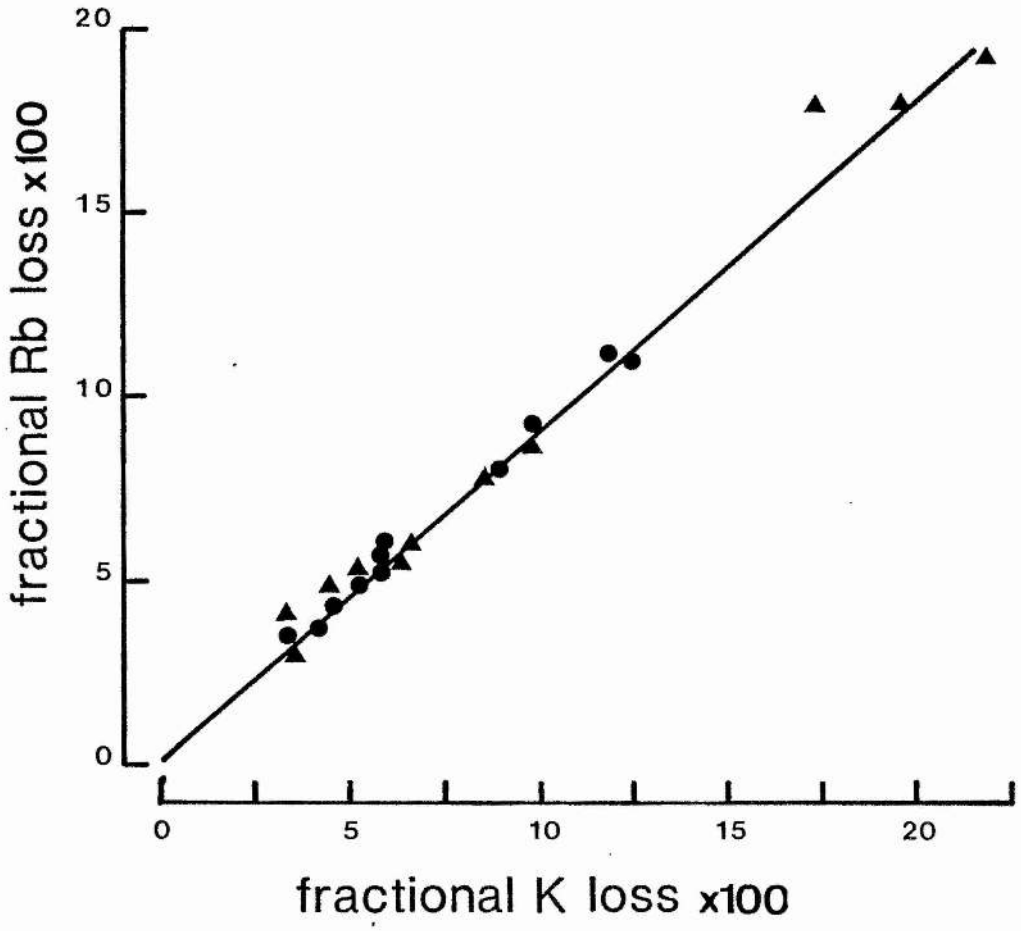

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
relative Rb influx
0
1
2
3
time (hours)

Fig 2.10 Validation of the use of ^{86}Rb to measure K^+ efflux from MDCK cell monolayers.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: fractional ^{42}K loss (x100). The suitability of ^{86}Rb as a tracer for K^+ efflux was measured using both ^{86}Rb and ^{42}K to, simultaneously, measure the same efflux rate from subconfluent cell monolayers of MDCK cells. Fractional K^+ loss was measured at 2 minute intervals under control (●) and adrenaline stimulated (▲) conditions. The solid line represents the least-squares regression line and was fitted by the equation:

$$y = 0.91(x) + 0.29$$

Both the slope of the line and the correlation coefficient (0.992) were highly significant ($P<0.001$).


20
15
10
5
0
fractional Rb loss x100
0
5
10
15
20
fractional K loss x100

CHAPTER 3

CATECHOLAMINE STIMULATION OF RHEOGENIC Cl^- SECRETION

INTRODUCTION

It is now well established that in many epithelia transepithelial Cl^- movements play an important role in the regulation of fluid and electrolyte balance in both health and disease (Schultz et al, 1974; Zadunaisky, 1978; Widdicombe & Welsh, 1980; Ferreira & Ferreira, 1981; Holmgren, 1981). With this in mind, over the last decade, a considerable research effort has been directed towards gaining an understanding of the molecular mechanisms involved in Cl^- absorption and Cl^- secretion by epithelia and it may be appropriate at this point to consider their findings so as to provide a general framework for the interpretation of the effects of catecholamines upon Cl^- transport by high resistance (strain I) MDCK cell monolayers.

Of the two transport processes (absorption and secretion) Cl^- absorption appears to be the more common, and certainly the better characterised, and is generally regarded a feature of "leaky" or moderately "leaky" epithelia such as; mammalian ileum (Schultz et al, 1974) and colon (Binder & Rawlings, 1973), gallbladder (Diamond, 1964; Frizzell et al, 1975), renal proximal tubule of Necturus (Spring & Kimura, 1978) and thick ascending limb of the loop of Henle (Greger, 1981). Frog skin and toad urinary bladder (Ussing et al, 1974) are notable exceptions to this rule being high resistance epithelia. In the majority of these epithelia it has become clear that Cl^- absorption is predominantly the result of a coupled 1:1 uptake of Na^+ and Cl^- in an electroneutral manner across the apical cell border, followed by a passive exit step for both ions across the basal-lateral cell border (fig 3.1). The uptake step for Cl^- is dependent upon the energy

inherent in the Na^+ gradient across the apical membrane generated by active Na^+ extrusion by the (Na^+ + K^+)-ATPase located on the basal cell border. Cl^- is accumulated 2-3fold above its electrochemical equilibrium (Field et al, 1978; Frizzell et al, 1979; Frizzell & Duffey, 1980). Experimental evidence in support of this model includes: (1) The demonstration of intracellular Cl^- activities above those expected from a passive distribution of Cl^- across the apical cell membranes, by chemical and radioisotope estimates (Frizzell et al, 1975), and subsequently with Cl^- selective microelectrodes (Armstrong et al, 1977; Spring & Kimura, 1978; Duffey et al, 1978; Garcia-Diaz & Armstrong, 1980). (2) Cl^- absorption is in general dependent upon Na^+ being present in the bathing media, and also a large fraction of the Na^+ flux is dependent upon the presence of Cl^- in the media. Measurement of the unidirectional Na^+ and Cl^- influxes across the apical border revealed a 1:1 coupling between the fluxes (Nellans et al, 1974; Cremashi et al, 1975; Frizzell et al, 1975). (3) It has been demonstrated that coupled influx of Na^+ and Cl^- across the apical cell membrane is responsible for generating a Cl^- accumulation by showing that intracellular accumulation of Cl^-, and net Cl^- absorption, could be abolished when the tissue was incubated in a Na^+-free medium (Duffey et al, 1975; Spring & Kimura, 1978; Garcia-Diaz & Armstrong, 1980). (4) Unlike frog skin where Cl^- absorption is electrically coupled to Na^+ transport (Ussing et al, 1974) and dependent upon a large transepithelial potential difference, coupled NaCl uptake by "leaky" epithelia, which generally support only a small transepithelial potential, is independent of potential. Spring & Kimura (1978) showed that in Necturus proximal tubule cells even when the apical membrane

potential was varied by over 60mV there was little apparent change in Cl^- accumulation. Conversely, removal of either Na^+ or Cl^- from the media, whilst inhibiting the uptake of the other ion, had no effect upon transmembrane electrical potential (Rose & Schultz, 1971; Schultz et al, 1974; Garcia-Diaz & Armstrong, 1980).

Although, in comparison to Cl^- absorption, our knowledge of epithelial Cl^- secretion is less complete, it is still apparent that in many secretory epithelia Cl^- movements can be accounted for by a single model (Frizzell et al, 1979, but see also Eveloff et al, 1978 and Simmons, 1981c). According to this model (fig 3.2): (1) Cl^- is accumulated across the basal-lateral membrane of secretory cells by an electroneutral NaCl coupled uptake mechanism using the energy inherent in the Na^+ gradient (Klyce & Wong, 1977; Ernst & Mills, 1977; Silva et al, 1977; Zadunaisky et al, 1979). The uptake mechanism displays similar properties to the uptake mechanism in absorptive epithelia. In dogfish rectal glands Cl^- is accumulated to ninefold that expected from a passive distribution of Cl^- across the basal-lateral cell membranes. Cl^- accumulation is ouabain-sensitive and dependent upon Na^+ ions in the bathing media (Silva et al, 1977). Direct evidence for coupled Na + Cl uptake across the basal-lateral membrane has been provided by Eveloff and co-workers (1978) who demonstrated that in basal-lateral membrane vesicles of rectal glands, in the absence of electrical driving forces Na^+ uptake was dependent upon the presence of Cl^- in the extra-vesicular fluid and that Na^+ uptake exhibited saturation kinetics when extra-vesicular NaCl, but not $NaNO_3$, concentrations were manipulated. These results suggest a limited number of Na + Cl transport sites on the basal-lateral membranes.

Coupled Na + Cl uptake was sensitive to inhibition by the diuretic furosemide. Similarly, furosemide was found to block Cl^- secretion in a number of intact tissues (Candia, 1973; Silva et al, 1977; Saito et al, 1980; Simmons, 1981c). (2) Active Cl^- secretion is subsequently the result of a hormone or secretagogue-induced increase in passive Cl^- exit across the apical cell membrane down a favourable electrochemical gradient (Silva et al, 1977). In the majority of secretory epithelia Cl^- secretion can be stimulated by agents which increase intracellular cyclic AMP: e.g. dibutryl cyclic AMP (Widdicombe & Welsh, 1980), adrenaline (Nagel & Reinach, 1980) or cholera toxin (Holmgren, 1981), or Ca^{2+} levels: e.g. the calcium ionophore A23187 (Frizzell, 1977). Cl^- movement across the apical cell membrane, in response to secretagogue stimulation, is rheogenic and is usually associated with a decrease in apical membrane electrical resistance (Klyce & Wong, 1977; Nagel & Reinach, 1980). Thus it would appear that both active Cl^- absorption and Cl^- secretion in a number of epithelia share many features in common: Both processes rely on the energy inherent in the sodium gradient across either cell membrane to accumulate chloride above its electrochemical equilibrium. Similarly, both processes employ a coupled Na^+ - Cl^- uptake mechanism although its exact nature is still a matter of debate (Turnberg et al, 1970; Nellans et al, 1973; Greger & Schlatter, 1981; Liedtke & Hopfer, 1982a) and a passive Cl^- exit step at the opposite membrane.

In several epithelial tissues Cl^- transport can be modified by adrenaline: In frog (Nagal & Reinach, 1980) and rabbit cornea (Klyce & Wong, 1977) adrenaline stimulates a net Cl^- secretion from the basal to apical membrane face. Similar findings are reported for frog skin

(Watlington, 1968; Tomlinson & Wood, 1978). In renal epithelia a catecholamine sensitive adenylate cyclase moeity has been reported to be localised in the late distal and early cortical collecting tubule of rabbit kidney (Morel et al, 1976) the physiological function of which may be to stimulate Cl^- reabsorption (Iino et al, 1981). Adrenergic stimulation is also thought to modulate renal sodium handling although both the mechanism and exact site of action remain unclear (Kim et al, 1980). In MDCK cells an adrenaline sensitive adenylate cyclase has been identified (Ishizuka et al, 1978) and addition of adrenaline is known to stimulate short circuit current in voltage-clamped epithelial monolayers (Richardson et al, 1981). The present series of experiments was designed to characterise and compare the effects of adrenaline upon ion transport by high resistance MDCK cell monolayers with the effects of adrenaline upon natural epithelia.

RESULTS

(A) The Effects of Catecholamines upon the Electrophysiological Parameters of MDCK Cell Monolayers.

The experiments described in this chapter were performed upon MDCK cells of between 66-74 serial passages, grown upon 2.5 cm diameter permeable filter supports and mounted into Ussing chambers with 1.76 cm^2 exposed monolayer area. The mean spontaneous potential difference generated by the epithelium was 1.2 $\pm$ 0.1 mV (n = 123) and the mean transepithelial electrical resistance was 3.5 $\pm$ 0.3 kΩ cm^2 (n = 123). Similar results have previously been reported for this strain (strain I) (Barker & Simmons, 1981; Simmons 1981a, and also chapter 2, section B(i)). Under basal conditions the monolayers support a small short circuit current (0.65 $\pm$ 0.02 uAmps.cm^{-2}) consistent with the magnitude of the open circuit potential and the small magnitude of net Na^+, K^+ and Cl^- fluxes (Table 3.1). A significant increase in short circuit current could be elicited with the addition of adrenaline (5 x 10^{-7} M) to the basal bathing solution (fig 3.3). The response was bi-phasic consisting of an initial peak in short circuit current of 28.6 $\pm$ 3.6 uAmps.cm^{-2} (n = 6) within the first two minutes, followed by a smaller (10.7 $\pm$ 1.6 uAmps.cm^{-2}) but maintained short circuit current response. In contrast, application of an equi-molar concentration to the apical bathing solution had no effect upon short circuit current (fig 3.3) mean results: basal S.C.C. = 2.6 $\pm$ 1.1 (n = 6), apical adrenaline = 1.6 $\pm$ 0.7 uAmps.cm^{-2} (n = 6) $P > 0.5$. Fig 3.3 also shows that the adrenaline dependent increase in short circuit could be rapidly reversed by repeated washing. An identical response could be produced if

adrenaline was re-applied after a five minute period (data not shown).

The observation that adrenaline was only effective from the basal bathing solution, coupled with the rapid onset and decay of the response, is evidence to suggest that the increased short circuit current response to adrenaline is the result of an interaction of adrenaline with surface receptors located exclusively on the basal-lateral membrane face, access to these receptors by apically applied adrenaline being limited by the effective permeability barrier afforded to the epitheleum by the apical tight junctions.

Table 3.2 demonstrates that the adrenaline dependent increase in short circuit current was the result of both an increased transepithelial open circuit potential, up by almost 30 mV in the presence of 2 uM adrenaline, and a concurrent decrease in transepithelial electrical resistance which falls by almost 75% within the first two minutes of the response and which may represent an increase in apical membrane conductance (see below and discussion). The lower maintained phase of the adrenaline response is accounted for by a recovery of transepithelial resistance and potential towards control levels (Table 3.2).

(B) Net Transepithelial Na^+, K^+ and Cl^- Fluxes; The Effects of Adrenaline.

In accordance with previous measurements made in this laboratory (Simmons, 1981a, 1981b; Barker & Simmons, 1981; Aiton et al, 1982), net Na^+, K^+ and Cl^- fluxes across high resistance MDCK (strain I) epithelium were not significantly different from zero (Table 3.1; $P > 0.5$ for each flux) under control conditions consistent with the

magnitude of the current flux equivalent recorded over the flux measurement period (Table 3.1).

Although addition of adrenaline (2 uM) to the basal bathing solution resulted in an increased short circuit current, expressed as current flux equivalent and averaged over the flux period for each ion (Table 3.1; $P < 0.01$ for each of the three flux measurements), it had no significant effect upon either the net Na^+ or net K^+ ion fluxes (Table 3.1) which under control conditions were not significantly different from zero ($P > 0.5$ for each ion). Adrenaline did however cause a significant reduction in the two uni-directional Na^+ fluxes ($P < 0.01$). In marked contrast to the lack of effect of adrenaline upon the net Na^+ and K^+ fluxes, adrenaline (2 uM) stimulated a significant net Cl^- movement from the basal to apical cell surfaces (secretion). Furthermore the magnitude of the net rheogenic Cl^- flux was of sufficient magnitude ($J.Cl_{net}$ = 0.46 umole.cm^{-2},hr^{-1}) to account for all of the adrenaline stimulated short circuit current response (current flux equivalent = 0.44 umole.cm^{-2}.hr^{-1}). A similar rheogenic secretion can be stimulated with exogenous ATP in this cell strain (Simmons, 1981b). Since adrenaline causes a significant decrease in transepithelial resistance (Table 3.2) which may reflect an increased paracellular conductance there is a possibility that net Cl^- secretion occurs via a paracellular pathway. However the significant decrease in Na^+ uni-directional fluxes in the presence of adrenaline (Table 3.1) suggests that Cl^- secretion is in fact transcellular and the conductance change is across either the apical or basal-lateral cell membrane since an increased paracellular shunt pathway would be associated with increased bi-directional ion fluxes (Frizzell et al, 1976; Barker & Simmons, 1981).

(C) The Ionic Dependence of Adrenaline Stimulated Cl^- Secretion

The dependence of the adrenaline stimulated short circuit current upon medium Na^+ ions is illustrated in fig 3.4. This shows that although Li^+ ions could support about 50% of the short circuit current generated by adrenaline in normal Krebs, both choline and tris were ineffective substitutes for sodium. In contrast, for ATP stimulated Cl^- secretion a marked cation dependence is not apparent (Simmons, 1981b). In a Na^+ free Krebs (choline substitution, apical and basal) 13 - 18% of the adrenaline response appeared to be Na^+ independent (fig 3.4). Partial replacement of medium Na^+ by choline revealed an upward curving, non-saturating, dependence of the short circuit current upon Na^+ ions (fig 3.5).

To further characterise the response, the effect of replacing either the apical or basal bathing Na^+ ions by choline was investigated. These experiments relied upon the fact that the transepithelial, and in particular the paracellular, Na^+ permeabilities were small and that Na^+ diffusion across the cell monolayer was negligible over the experimental period. This was confirmed by flame photometry of the appropriate bathing solution subsequent to the experimental period. To try to attain true Na^+ - free conditions, especially in the unstirred layers surrounding the epithelium, the monolayers were incubated for ten minutes in the appropriate Na^+ - free buffer, followed by a final solution change immediately prior to the addition of adrenaline. Longer pre-incubations led to a decrease in transepithelial resistance indicating that Na^+ replacement leads to non-specific changes in monolayer integrity. Fig 3.6 shows that replacement of apical Na^+ by choline had no significant effect ($P > 0.5$) upon the magnitude of the adrenaline

stimulated short circuit current. In contrast, a Na^+ - free basal bathing solution resulted in a large (80%) inhibition of the adrenaline response. As with bi-lateral Na^+ - replacement, a significant proportion of the short circuit response was Na^+ independent (fig 3.5, fig 3.6).

To characterise the dependence of the adrenaline response upon Cl^- ions a similar series of experiments were carried out. The ability of a series of anion replacements to support the adrenaline stimulated short circuit current was examined (fig 3.7). Whilst Br^- was partially able to replace Cl^- thiocyanate, isethionate and NO_3 were ineffective replacements. The relative potency of anions to support the adrenaline response was therefore: $Cl^- > Br^- \gg SCN^- > isethionate^- > NO_3^-$. Partial replacement of medium Cl^- ions with NO_3^- revealed a saturable relationship between the magnitude of the current response and the bathing medium Cl^- concentration over the range of 20-180 mM Cl^-. The apparent K_m for the Cl^- dependence of the adrenaline response was 65mM (fig 3.8). Although these results suggest the transport mechanism has a high selectivity for Cl^- as the transported anion, a non-specific effect of low-chloride media upon Ca^{2+} ion chelation (see below) cannot be excluded (Kenyon & Gibbons, 1977).

Using the same experimental protocol as outlined for Na^+ ions (see above) the effects of uni-lateral Cl^- replacement of either the apical or basal bathing solutions was investigated. Replacement of the apical bathing solution Cl^- with isethionate led to a slight but non-significant (upon grouped data) increase in the adrenaline stimulated short circuit current compared to Cl^- - containing media (fig 3.6). This slight increase may have resulted from a more favourable gradient

for Cl^- efflux across the apical cell membrane. Replacement of the basal Cl^- ion concentration, again with isethionate, resulted in a marked inhibition of the adrenaline response (76%), similar to the inhibition achieved by removal of basal Na^+ (fig 3.6). On the basis of a similar proportion of the short circuit current being dependent upon the presence of both Na^+ and Cl^- in the basal bathing solution and of the lack of effect of apical cation and anion replacements, these results may imply the existence of a coupling of Na^+ and Cl^- transport across the basal-lateral cell membrane (see discussion and Frizzell & Duffey, 1980; Eveloff et al, 1978).

In frog skin it has been suggested that a component of the short circuit response to adrenaline is the result of Na^+ re-absorption across the apical cell membrane (Tomlinson & Wood, 1978), although subsequently, cells in which Cl^- secretion accounts for the total short circuit current have been identified (Mills et al, 1982). That part of the adrenaline response of MDCK cells results from sodium re-absorption appears unlikely since replacement of apical Na^+ by choline had no significant effect upon short circuit current (fig. 3.6).

From fig 3.4 it is clear that the adrenaline response is composed of two main phases: an initial transient phase which includes the peak response and a lower magnitude maintained phase. All ion replacements so far described have concentrated upon effects upon the initial phase of the response - one minute following adrenaline addition, so although it would appear likely on the basis of the average flux equivalent data (Table 3.1), it was important to demonstrate that similar responses could be obtained on the maintained phase of the short circuit current response. This was achieved by comparing the effect of ion replacement

upon the short circuit current response one minute and five minutes after the addition of adrenaline. For basal Na^+ replacement with choline the short circuit current was reduced from 35.8 ± 5.0 (n = 7) to 6.4 ± 1.6 uAmps.cm^{-2} (n = 7) at plus one minute (an 83% inhibition) and from 9.7 ± 0.4 (n = 7) to 1.2 ± 0.3 uAmps.cm^{-2} (n = 7) at plus five minutes. Similarly for basal Cl^- replacement with isethionate the adrenaline stimulated short circuit current was reduced from 28.9 ± 4.1 (n = 6) to 6.5 ± 0.9 uAmps.cm^{-2} (n = 6) at plus one minute (a 78% inhibition) and from 7.9 ± 1.5 (n = 6) to 0.9 ± 0.3 uAmps.cm^{-2} (n = 6) after five minutes (an 88% inhibition), suggesting that the ionic basis of the short circuit current response to adrenaline is identical over the two phases. Similar results can be obtained for bi-lateral Na^+ and Cl^- replacements.

(D) Action of Certain Pharmacological Agents upon the Adrenaline Stimulated Cl^- Secretion

An insight into the molecular mechanisms behind the adrenaline stimulated short circuit current can be gained with the selective use of drugs with known sites and mechanisms of action in natural epithelia. Thus amiloride a blocker of passive Na^+ entry across the apical cell membrane of a number of epithelia (Erlij & Smith, 1973; Lewis et al, 1976; Cuthbert & Shum, 1974) was used to investigate the contribution passive Na^+ reabsorption made to the adrenaline stimulated short circuit current. Table 3.3 shows that amiloride, in fact, had no effect upon the adrenaline response when added to the apical bathing solution. This result is consistent with the lack of effect of a Na^+-free apical bathing solution (fig 3.6). Adrenaline-stimulated Cl^-

secretion across frog skin has also been reported to be amiloride insensitive (Tomlinson & Wood, 1978).

Similarly the effects of three inhibitors of Cl^- transport in epithelia and other tissues were tested for their ability to inhibit the adrenaline stimulated Cl secretion. The stilbene; SITS, an effective inhibitor of coupled Cl^- - HCO_3^- (or Cl^- - OH^-), exchange fluxes in a number of epithelial tissues (Leslie et al, 1973; Liedtke, 1978; Liedtke & Hopfer, 1982) had no significant effect upon the adrenaline response when added to either bathing solution at a concentration of 10 uM. In contrast, the adrenaline response was sensitive to inhibition by phloretin (Cousin & Motais, 1977); roughly 45% of the adrenaline sensitive short circuit current was blocked by 1×10^{-4} M phloretin in either the apical or basal bathing solution (Table 3.3). Similarly the loop diuretic furosemide was able to inhibit over 70% of the adrenaline response when added to the basal-lateral bathing solution. In contrast to phloretin, apically applied furosemide was ineffective (Table 3.3). To elucidate the mode of action of furosemide its effects upon the adrenaline stimulated open circuit potential and apical membrane conductance were investigated (Table 3.2). It is evident from these measurements that the inhibitory actions of furosemide are predominately the result of an inhibition of the electrogenic component of the adrenaline response; the open circuit potential declines over a five minute period to only 30% of its steady state value in the presence of adrenaline alone. Furosemide has no significant effect upon the adrenaline stimulated increase in transepithelial conductance. This data is consistent with an inhibitory action of furosemide directly upon ion transport rather than an effect upon other cellular events

such as activation of adenylate cyclase. It is also apparent from Table 3.2 and Table 3.3 that a significant proportion of the adrenaline dependent short circuit current response was furosemide insensitive.

(E) Adrenoreceptor Pharmacology

In order to establish which type of receptor was responsible for the adrenaline stimulated short circuit current, the ability of several agonists to stimulate short circuit current was tested. As well as adrenaline (fig 3.9) short circuit current was also stimulated, in a dose dependent manner, by isoprenaline (fig 3.10) and by noradrenaline (fig 3.11). Analysis of these curves by Probit analysis gives apparent affinity constants for their ability to stimulate short circuit current of isoprenaline (EC_{50} = 1.5 $\pm$ 0.2 x 10^{-8}M) $\geqslant$ adrenaline (EC_{50} = 3.1 $\pm$ 0.3 x 10^{-8}M) $\gg$ noradrenaline (EC_{50} = 6.0 $\pm$ 0.2 x 10^{-6}M), consistent with stimulation of beta-adrenoreceptors of the $beta_2$- subtype (Lands, 1967; Rugg & Simmons, 1982) although the difference in affinities between isoprenaline and adrenaline are less than might be expected. Also the response differs from a normal beta-receptor response in that, although isoprenaline has the greatest apparent affinity for the receptors (see above), the efficacy with which isoprenaline stimulates short circuit current was less than 20% that of either adrenaline or noradrenaline (compare figs 3.9, 3.10, 3.11) - that is to say that the maximum short circuit current stimulation seen with isoprenaline was 6.8 $\pm$ 0.6 uAmps.cm^{-2} (n = 6) compared with 35 $\pm$ 2.2 (n = 5) and 32 $\pm$ 2.6 (n = 4) uAmps.cm^{-2} for adrenaline and noradrenaline respectively. Since isoprenaline is a specific beta-adrenoreceptor agonist and not known to be a partial agonist (Lees, 1981) these results imply that a

substantial proportion of the adrenaline and noradrenaline stimulated short circuit current must result from activation of alpha-adreno-receptors by these drugs. Evidence in support of this proposal is presented in fig 3.12 which shows that the adrenaline-stimulated short circuit current is composed of two major pharmacologically distinct components; a propranolol sensitive beta-receptor mediated short circuit current and phentolamine sensitive alpha-receptor mediated component. In the presence of both drugs adrenaline was ineffective in stimulating short circuit current.

To test the relative contributions of alpha- and beta-receptor activation of short circuit current the efficacy of a specific alpha-adrenoreceptor agonist phenylephrine (Lees, 1981) was compared with those of isoprenaline and adrenaline. The results (fig 3.13) show that even at relatively high concentrations (1×10^{-4}M) phenylephrine was not a potent stimulator of short circuit current. However, addition of phenylephrine plus isoprenaline, in effect activation of both alpha- and beta-receptors, generated a short circuit current response of similar magnitude to an adrenaline response, and far greater than the maximum response than might have been generated through additivity alone. A similar attempt to investigate the inter-relationships between alpha- and beta-stimulation and short circuit current was made by blocking either the alpha-receptor with phentolamine, or the beta-receptor with propranolol and measuring the current generated in the presence of adrenaline. The results of this experiment (fig 3.13) show that blockade of either receptor resulted in a marked inhibition of the adrenaline response and the magnitude of the inhibition far exceeded the maximum current generated by either isoprenaline or phenylephrine (fig

3.13). Although these experiments were by necessity rather crude the data clearly demonstrates that the majority of the short circuit current generated by adrenaline, or high doses of noradrenaline, results from the interaction of these drugs with both receptor types and is not directly attributable to stimulation of either receptor. A similar potentiation of a response by activation of both alpha- and beta-receptors has been described in liver slices (Jenkinson & Koller, 1977). In both cases the site and mechanism by which alpha-agonists potentiate a beta-response are as yet unclear but may involve an effect upon membrane sensitivity to cyclic nucleotides (Jenkinson & Koller, 1977; Jenkinson et al, 1978). Another possible candidate may be a synergy between intracellular cyclic AMP and free intracellular Ca^{2+} ion levels in mediating the cellular actions of adrenaline (but see Chapter 5 and Ilundain & Naftalin, 1979).

DISCUSSION

In this chapter we have investigated the effects of catecholamines upon ion transport across high resistance epithelial monolayers of MDCK cells. From the data presented in Table 3.1 it can be concluded that the major proportion of adrenaline stimulated short circuit current is carried by a rheogenic Cl^- secretion from the basal-lateral to the apical cell surfaces. Adrenaline has no significant effect upon the net transepithelial fluxes of either Na^+ or K^+ which remain not significantly different from zero. Similar effects of adrenaline upon Cl^- transport have been reported for a number of natural epithelia (Klyce et al, 1973; Watlington et al, 1977; Silva et al, 1977). That catecholamine stimulation of Cl^- secretion in MDCK cells results from the interaction of adrenaline with both alpha- and beta-adrenoreceptors is suggested from the following data: (a) The affinities of various adrenoreceptor agonists is consistent with activation of a beta-adrenoreceptor (Lands, 1967); (b) Although isoprenaline has the highest affinity for the ability to stimulate short circuit current it has a low efficacy compared to adrenaline or noradrenaline. Similarly activation of alpha-receptors alone with phenylephrine results in only a small short circuit current response. Application of isoprenaline plus phenylephrine results in a greater stimulation of short circuit current than might be expected from additivity alone; (c) Adrenaline stimulated short circuit current can be partly inhibited by propranolol, the significant short circuit current remaining being sensitive to inhibition by the alpha-receptor antagonist phentolamine. The level of inhibition achieved with either phentolamine or propranolol exceeded

the maximum short circuit current that could be stimulated by either isoprenaline or phenylephrine.

These results suggest that the maximum stimulation of Cl^- secretion observed with adrenaline results from the stimulation of both alpha- and beta-adrenoreceptors located upon the basal-lateral cell membranes. However it seems likely that the potentiating effect of alpha-adrenoreceptor occupation is not the result of a direct alpha-receptor mediated stimulation of Cl^- secretion *per se* through a rise in intracellular free Ca^{2+} (Frizzell, 1977; Ilundain & Naftalin, 1979) but rather as an indirect result of alpha-stimulation. In a number of tissues, including epithelia and MDCK cells (see Chapter 5), stimulation of alpha-adrenoreceptors results in a Ca^{2+}- dependent increase in membrane K^+ permeability (Jenkinson *et al*, 1978; Putney, 1979) which can be associated with a hyperpolarisation of the membrane potential (Karashima, 1981). Evidence presented in this chapter suggests that Cl^- secretion in MDCK cells is achieved by a similar mechanism to secretion in several natural epithelia i.e. coupled electroneutral NaCl accumulation across the basal-lateral cell membrane followed by a passive energetically downhill Cl^- exit step across the apical cell membrane (see below), so that a possible mechanism by which stimulation of alpha-receptors potentiates Cl^- secretion may be via an alpha-receptor mediated stimulation of cellular K^+ efflux hyperpolarising the membrane potential and increasing the favourable electrochemical gradient for the passive Cl^- exit step across the apical membrane (McLennan *et al*, 1980; Moreto *et al*, 1981). In this context an ATP stimulated hyperpolarisation of the membrane potential of MDCK cells has recently been demonstrated (N.L. Simmons; unpublished observations).

Many features of the adrenaline-stimulated Cl^- secretion by MDCK cells are common to secretagogue-induced Cl^- secretion in other natural epithelia. In frog (Nagel & Reinach, 1980) and rabbit (Klyce & Wong, 1977) corneal epithelia, and in frog skin (Tomlinson & Wood, 1978) stimulation of Cl^- secretion is usually accompanied by an increase in apical membrane conductance. In Table 3.1 we demonstrate that addition of adrenaline to MDCK cell monolayers, mounted without edge damage (see methods section), results in a similar increase in transepithelial conductance. In natural high resistance epithelia, mounted without edge damage, up to 95% of the transepithelial resistance is across the apical cell aspects, and transjunctional resistance is thought to be greater than the resistance of the apical cell membrane (Higgins et al, 1975; Fromter et al, 1977; Higgins et al, 1977). MDCK cell monolayers also display a high electrical resistance/low potential profile (Chapter 2 and Simmons, 1981a), suggesting that the apical membrane resistance is relatively high in comparison to the basal-lateral membrane resistance. If this is indeed the case then the adrenaline stimulated increase in transepithelial conductance might be concluded to reflect an increased apical membrane conductance as has been demonstrated in natural Cl^- secretory epithelia (see above).

Cl^- secretion in a number of epithelia, such as: rabbit ileum and colon (Al-Awquati et al, 1974; Frizzell et al, 1979), fish gills (Degnan et al 1977), shark rectal glands (Silva et al, 1977), toad ciliary epithelium (Saito et al, 1980), dog tracheal epithelium (Davis et al, 1977) and frog cornea (Candia et al, 1981), exhibit a similar pharmacological sensivity inhibitable by the loop diuretics furosemide and ethacrynic acid, and also by phloretin. For MDCK epithelium a substantial part

of the adrenaline and ATP (Simmons, 1981a) stimulated Cl^- secretion is sensitive to inhibition by furosemide, ethacrynic acid and phloretin. Furthermore the use of the Cl^- transport inhibitors furosemide and phloretin suggests the existence of two pharmacologically distinct Cl^- transport mechanisms, one upon the basal-lateral membranes and one upon the apical membranes. Thus, whereas phloretin inhibits Cl^- secretion when applied to either bathing solution, furosemide is only effective from the basal-lateral surface. A similar site of action for furosemide has been reported for other Cl^- secretory epithelia (Candia, 1973; Eveloff et al, 1978; Davis et al, 1977) and is in contrast to an apical site of action found in Cl^- absorptive epithelia such as thick ascending limb of the loop of Henle (Burg et al, 1973; Greger, 1981) and small intestine (Humphreys, 1976).

The ability of various ions to support the adrenaline stimulated Cl^- secretion ($Cl > Br \gg I > NO_3$) and the apparent K_m of the transport process for Cl (65 mM) is similar to that for ATP-stimulated Cl^- secretion in MDCK cells (Simmons, 1981c) and for Cl^- transport in dog tracheal epithelium (Widdicombe et al, 1979), and corresponds to a selectivity isotherm for anions of type 4 (Wright & Diamond, 1977), suggesting that the rate limiting step for Cl^- is hydrophobic, similar to mammalian small intestine (Holman & Naftalin, 1979). The dependence of adrenaline stimulated Cl^- secretion upon extracellular Na^+ is also reminiscent of other Cl^- secretory epithelia such as shark rectal gland (Silva et al, 1977), fish gills (Degnan et al, 1977) and dog tracheal epithelium (Widdicombe & Welsh, 1980). Since Cl^- secretion comprises of Cl^- transport across two cellular membranes, assuming a transcellular route for Cl^- secretion, an attempt has been made to characterise the

dependence of such transport upon Na^+ and Cl^- in the appropriate bathing solution. This experiment requires that transepithelial ion diffusion is minimal over the experimental period. For bulk solution Na^+ this has been demonstrated by flame photometry although it is possible that Na^+- or Cl^-- free conditions may not have been obtained within small intracellular compartments. Notwithstanding this possible error, the Na^+ and Cl^- dependence of the adrenaline short circuit current have both been demonstrated to be due solely to an effect upon the basal-lateral cell membrane. Removal of apical Cl^- or Na^+ was without effect upon the magnitude of the adrenaline stimulated Cl^- secretion.

In the last few years a model of epithelial Cl^- secretion has been proposed by several groups (Silva et al, 1977; Eveloff et al, 1978; Field, 1978; Frizzell et al, 1979; Frizzell & Duffey, 1980) to account for observations made on a number of epithelial tissues. The model proposes that Cl^- is accumulated into the cell, above its electrochemical equilibrium, by a coupled diuretic sensitive Na + Cl cotransport mechanism at the basal-lateral membrane, utilising the energy inherent in the Na^+ gradient established by active Na^+ extrusion across the basal-lateral cell aspects by the $(Na^+ + K^+)$-ATPase. Cl^- secretion is then envisaged to result from a secretagogue induced increase in apical membrane Cl^- conductance and a passive downhill movement of Cl^- across the apical cell membrane. A similar mechanism has been proposed to explain Cl^- secretion in MDCK cells (Simmons, 1981c). Evidence in favour of a similar model applying to adrenaline stimulated Cl^- secretion includes: (a) Secretion is dependent upon both Cl^- and Na^+ in the basal-lateral but not the apical bathing solution. Both these observations are consistent with a coupled uptake of Na + Cl at the

basal-lateral membrane and a passive electrogenic exit step at the apical cell border. (b) That furosemide is only effective at the basal-lateral membrane is also consistent with this model where it inhibits Cl^- accumulation into the cell. As shown in Table 3.2 furosemide has no effect upon the secretagogue-induced increase in membrane conductance at the apical membrane.

It may at this point be worthwhile to speculate as to the nature of the proposed Na + Cl coupled accumulation mechanism at the basal-lateral cell membranes. One mechanism for the entry step for Cl^- across the basal-lateral membrane is a tightly coupled Na+ Cl cotransport mechanism (Nellans et al, 1974; Eveloff et al, 1978; Field, 1978) akin to Na-dependent solute transport (Alvarado, 1975). An alternative model is that NaCl uptake is the net result of two coupled ion exchange mechanisms: Na^+ for H^+ and Cl^- for HCO_3^- (or OH^-) such as has been proposed in small intestine (Turnberg et al, 1970; Liedtke & Hopfer, 1982a) and renal epithelia (Warnock & Yee, 1981). However, recently it has been proposed that a variety of ion co-transports (e.g. $Na^+ + Cl^-$, $K^+ + Cl^-$ and $K^+ : K^+$ exchange) are functional expressions of a single diuretic sensitive ($Na^+ + K^+ + Cl^-$) cotransport mechanism (Chipperfield, 1980; Geck, 1980). For MDCK cells a diuretic sensitive co-transport system has recently been identified (Aiton et al, 1981a, 1981b). Thus we have three possible mechanisms by which Cl^- can be accumulated into the cell across the basal-lateral cell membranes: (1) coupled Na + Cl cotransport; (2) coupled Na + K + Cl cotransport and (3) $Na/H + Cl/HCO_3$ exchange. The existence of these mechanisms has been rigorously tested in only a few Cl^- transporting epithelia (Eveloff et al, 1978; Eveloff et al, 1980; Liedtke & Hopfer, 1982b).

In all models the Cl^- exit step at the opposite membrane is thought to be via a passive Cl^- channel the conductance of which may be modified by cyclic nucleotides or Ca^{2+}.

It is clear however that measurements of intracellular potential and Cl^- concentrations are required to verify the major features of the proposed models of Cl^- secretion in MDCK cells, especially since Cl^- activities above those expected from an equilibrium distribution of Cl^- are not always seen in Cl^- transporting epithelia (Gerenscer & White, 1981).

TABLE 3.1

Summary of the transepithelial Na^+, K^+ and Cl^- fluxes across MDCK monolayers voltage clamped to zero p.d. J_{a-b} denotes the flux of an ion from the apical to basal surfaces of the cell layer. J_{b-a} denotes the reverse flux. Values of short circuit current are mean values recorded continually throughout the flux measurement period at five minute intervals.

Significantly different from control values:

N.S.	Non significant
a	$p < 0.02$
b	$p < 0.01$

ION	ADRENALINE uM	N	J_{a-b} umole.cm^{-2}.hr^{-1}	J_{b-a} umole.cm^{-2}.hr^{-1}	J_{net} umole.cm^{-2}.hr^{-1}	SCC umole.cm^{-2}.hr^{-1}
Na 137mM	0	11	1.28 ± 0.07	1.16 ± 0.03	0.14 ± 0.14	0.03 ± 0.01
			b	b	N.S.	b
	2	11	0.69 ± 0.14	0.54 ± 0.10	0.18 ± 0.07	0.38 ± 0.06
K 5.4mM	0	13	0.21 ± 0.09	0.16 ± 0.06	0.04 ± 0.04	0.04 ± 0.01
			N.S.	N.S.	N.S.	b
	2	11	0.14 ± 0.05	0.11 ± 0.04	0.03 ± 0.02	0.53 ± 0.11
Cl 160mM	0	7	0.82 ± 0.24	0.74 ± 0.34	0.08 ± 0.16	0.04 ± 0.01
			N.S.	a	a	b
	2	11	1.29 ± 0.15	1.75 ± 0.24	-0.46 ± 0.06	0.44 ± 0.05

TABLE 3.2

The effects of adrenaline (2uM) and adrenaline plus furosemide (1×10^{-4}M) upon open circuit potential and transepithelial electrical resistance across epithelial monolayers of MDCK cells. To optimise the accuracy of the resistance measurement 9.62 cm^2 area monolayers were employed and mounted without edge damage in the improved chamber design (see methods section).

	POTENTIAL (mV)			RESISTANCE ($k\Omega.cm^2$)		
TIME	CONTROL	ADRENALINE	ADRENALINE + FUROSEMIDE	CONTROL	ADRENALINE	ADRENALINE + FUROSEMIDE
2 Min	4.2 ± 0.8	31.4 ± 1.6	21.3 ± 2.5	9.1 ± 1.4	2.5 ± 0.3	3.6 ± 0.6
5 Min	4.3 ± 1.0	31.9 ± 0.7	8.5 ± 1.6	10.1 ± 1.8	3.9 ± 0.5	4.8 ± 0.9
10 Min	4.3 ± 0.9	30.9 ± 2.1	7.8 ± 1.9	10.5 ± 1.8	4.4 ± 0.6	5.0 ± 0.9

TABLE 3.3

The effect of inhibitors of ion transport upon the adrenaline stimulated (5×10^{-7}M) short circuit current. Amiloride, phloretin and SITS were added for a ten minute preincubation period. The adrenaline dependent increase in short circuit current was measured one minute following adrenaline stimulation. Furosemide was added six minutes subsequent to adrenaline stimulation and the short circuit current recorded after a further five minutes; control values are obtained eleven minutes after adrenaline stimulation. (a) and (b) denote apical or basal additions respectively. Figures in parentheses are the number of observations. Errors are $\pm$ S.E.M.

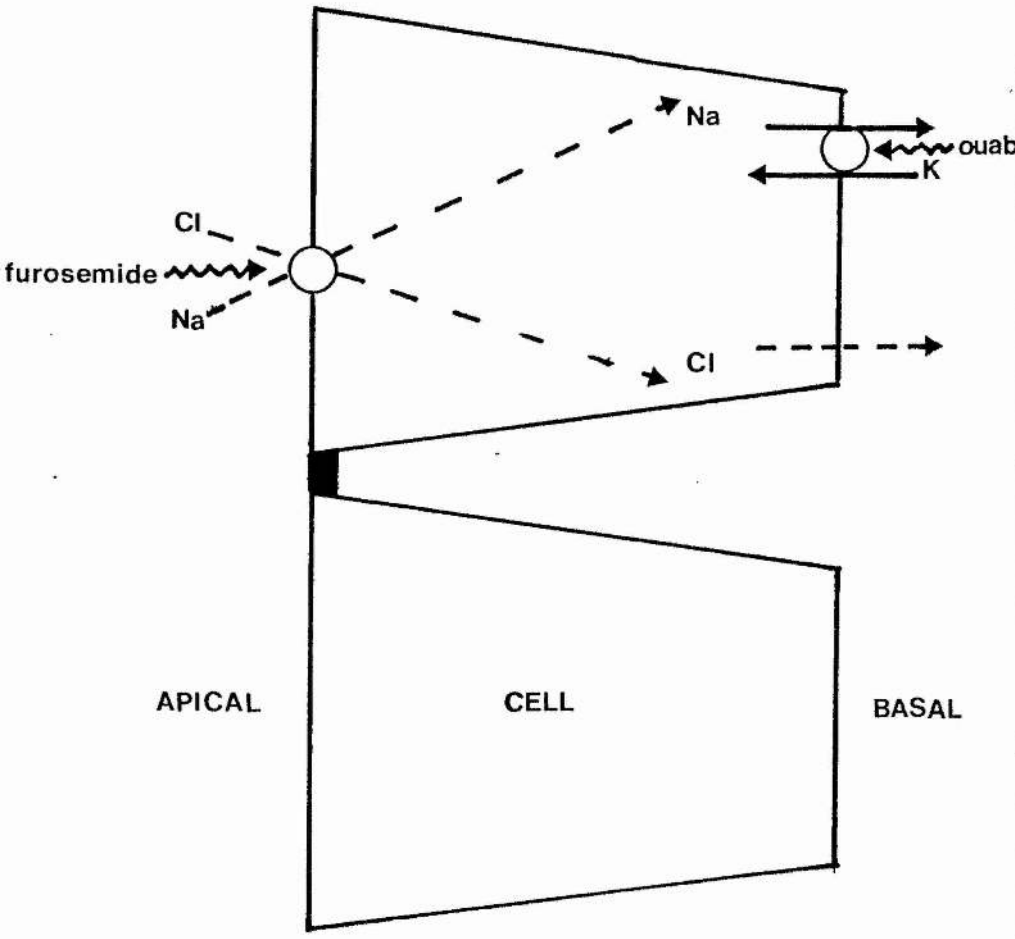
Inhibitor	Control response uAmps.cm^{-2}	Test response uAmps.cm^{-2}	p value of difference
Amiloride (a) $1x10^{-4}$M	34.6 ± 4.6 (4)	26.8 ± 3.3 (4)	N.S.
Phloretin (a) $1x10^{-4}$M	30.2 ± 3.7 (7)	16.4 ± 2.6 (7)	0.01
Phloretin (b) $1x10^{-4}$M	20.0 ± 4.0 (5)	11.4 ± 1.8 (5)	0.01
SITS (a) 10uM	27.7 ± 2.2 (9)	30.5 ± 1.4 (6)	N.S.
SITS (b) 10uM	27.7 ± 2.2 (9)	32.6 + 1.2 (6)	N.S.
Furosemide (a) $1x10^{-4}$M	8.9 ± 0.9 (5)	7.9 ± 0.8 (5)	N.S.
Furosemide (b) $1x10^{-4}$M	6.8 ± 0.6 (5)	2.4 ± 0.4 (5)	0.001

Fig 3.1 A working model for Cl^- transport in absorptive epithelia.

Cl^- is accumulated into the cell across the apical cell membrane against its electrochemical gradient by a coupled secondary active Na + Cl transport mechanism. This uptake mechanism is sensitive to inhibition by the loop diuretic; furosemide. Absorpted Na^+ is actively extruded from the cell across the basal-lateral cell membrane by the Na^+-pump. The Cl^- exit step across the basal-lateral membrane is thought to be passive. Active ion movements in this diagram is indicated by solid lines, passive ion movement by broken lines.

Fig 3.2 A working model for Cl^- transport in secretory epithelia.

Cl^- is accumulated into the cell across the basal-lateral cell membrane against an electrochemical gradient, by a 2ndry active Na + Cl coupled mechanism using the energy inherent in the Na^+ gradient. This mechanism is sensitive to inhibition by the loop diuretic; furosemide. Na^+ is actively extruded across the basal-lateral cell membrane by the Na^+-pump. The Cl^- exit step across the apical membrane is passive, and via a cAMP activated membrane conductance pathway. Active ion movement in this diagram is indicated by solid lines, passive ion movement by broken lines.


Na
ouab
K
Cl
furosemide
Na
Cl
APICAL
CELL
BASAL

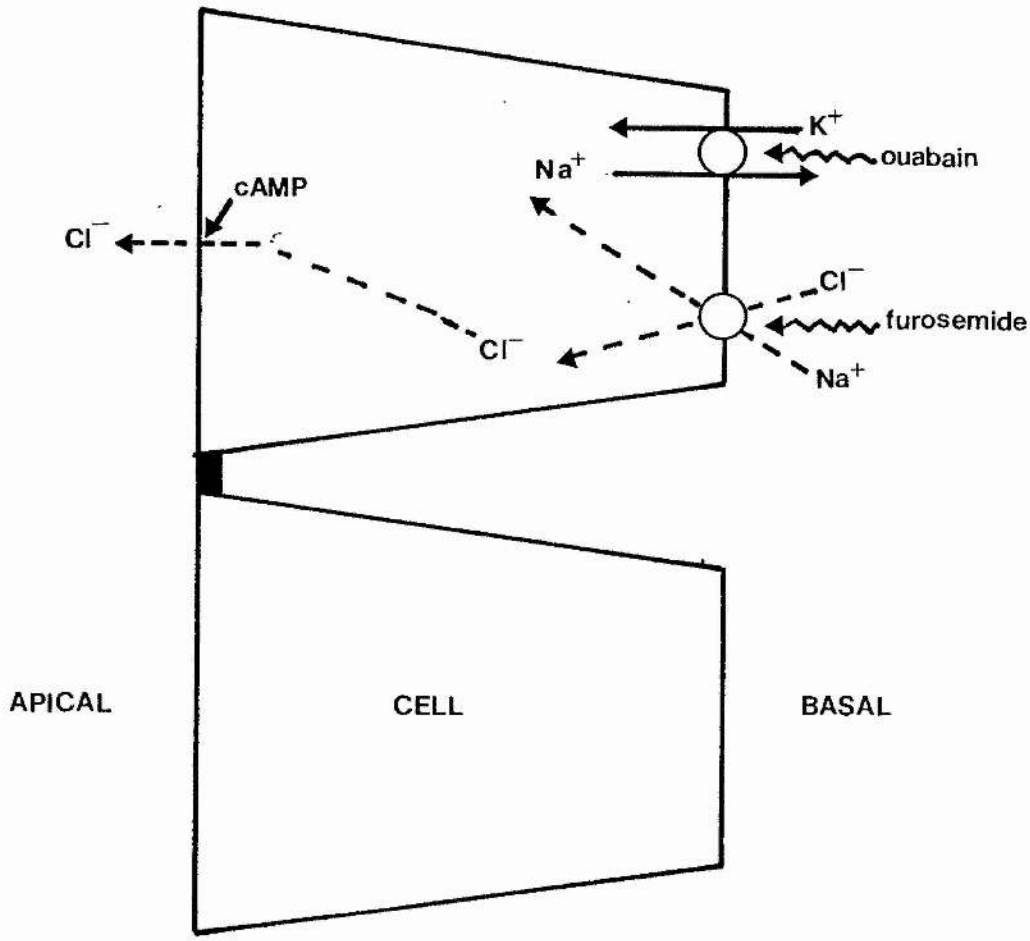

K+
ouabain
Na+
cAMP
Cl−
Cl−
Cl−
furosemide
Na+
APICAL
CELL
BASAL

Fig 3.3 The effect of adrenaline upon the short circuit current maintained by high resistance MDCK cell monolayers.

Abscissa: time (minutes). Ordinate: short circuit current (uAmps.cm^{-2}). Adrenaline (5×10^{-7}M) was added to the apical (A) and then to the basal-lateral bathing solutions. The adrenaline stimulated short circuit current was rapidly reversed by repeated washing (replacement of both bathing solutions x4). The basal short circuit current was 1.6 uAmps.cm^{-2} and the transepithelial resistance, prior to adrenaline addition was 4.2 kΩ cm^2. The data is from a single representative experimental trace.

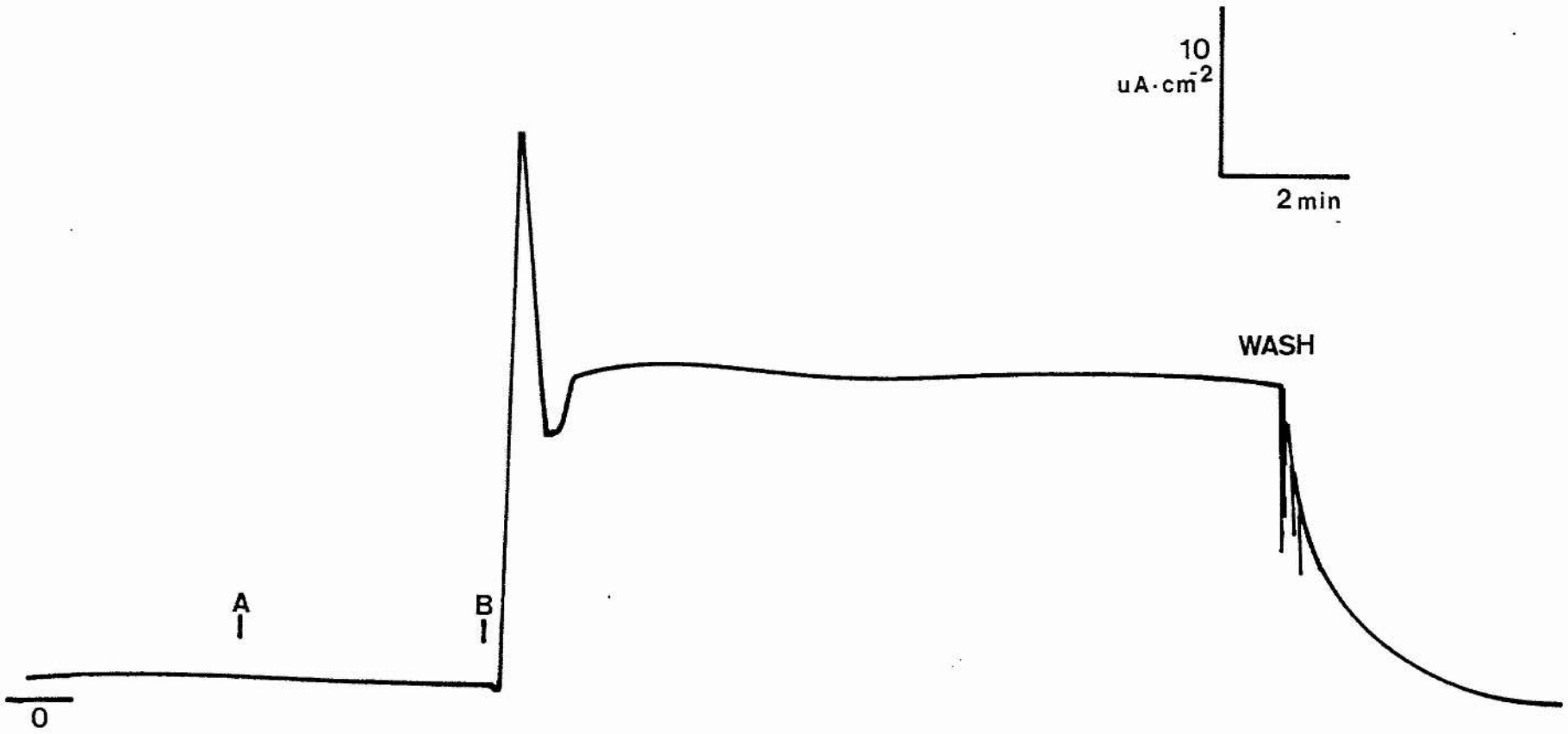

10
uA·cm-2
2 min
WASH
A
B
0

Fig 3.4 The Na^+ dependence of the adrenaline stimulated short circuit current response.

Ordinate: Short circuit current (uAmps.cm^{-2}). Epithelial monolayers were preincubated for 10 minutes in the appropriate test solution (apical and basal-lateral replacement). The short circuit response to 2 x10^{-7}M adrenaline was recorded 1 minute after adrenaline addition. The figures in parentheses are the number of separate observations of each condition. Errors are expressed as $\pm$ S.E.M.

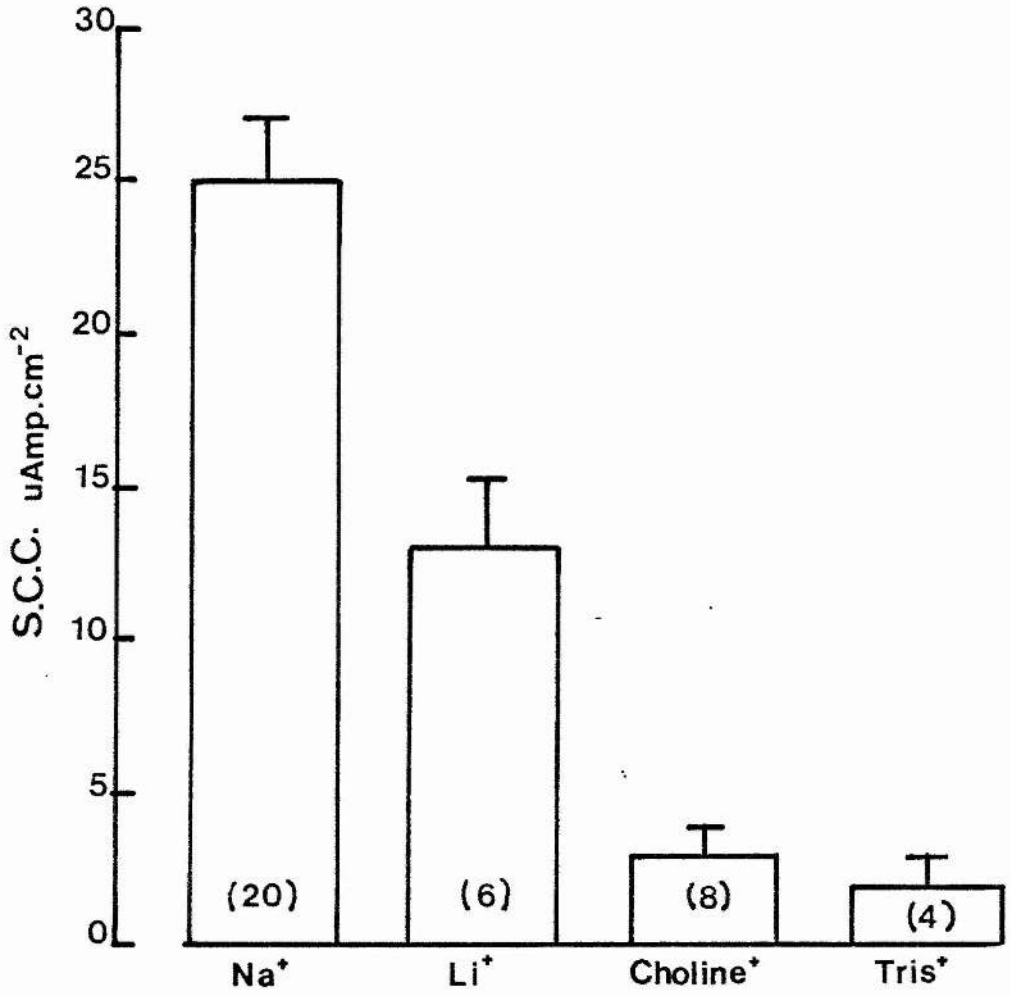

30
25
20
15
10
5
0
S.C.C. uAmp.cm-2
(20)
(6)
(8)
(4)
Na+
Li+
Choline+
Tris+

Fig 3.5 The effect of varying Na^+ upon the adrenaline stimulated short circuit current response.

Abscissa: Na^+ concentration (mM). Ordinate: short circuit current (uAmps.cm^{-2}). The extracellular Na^+ concentration was varied over the range 0-140 mM by isosmotic replacement of Na^+ with choline. Cell monolayers were preincubated for 10 minutes (apical and basal-lateral replacement), before addition of 5 x10^{-7}M adrenaline. The adrenaline stimulated short circuit current was then recorded after 1 minute. The results are expressed as the mean $\pm$ S.E.M. of 6 separate observations.

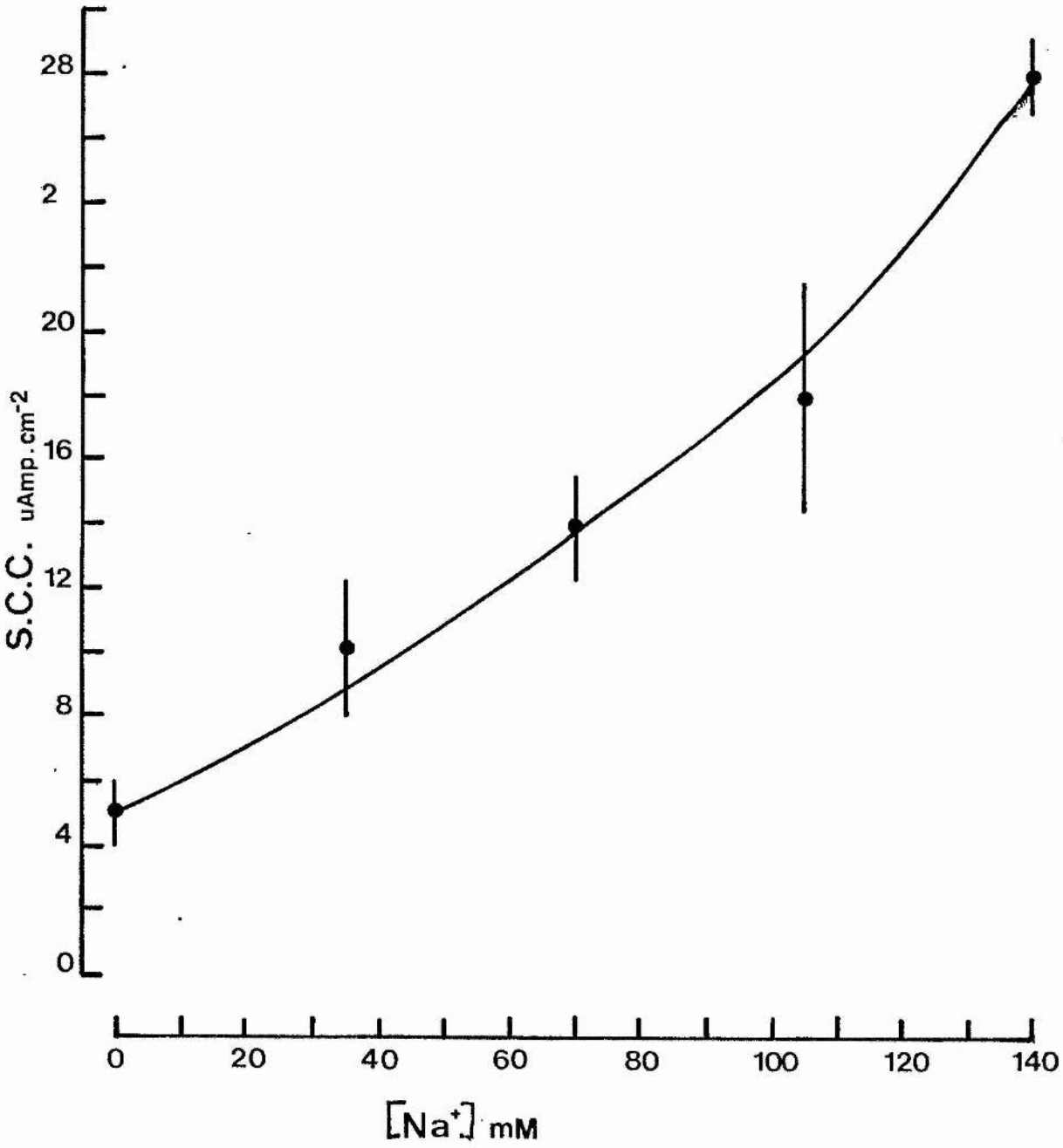

28
2
20
16
12
8
4
0
S.C.C. uAmp.cm-2
0
20
40
60
80
100
120
140
[Na+] mM

Fig 3.6 The effect of either apical or basal-lateral Na^+ or Cl^- replacement upon the adrenaline stimulated short circuit current response. Ordinate: short circuit current (uAmps.cm^{-2}). To test the sidedness of the Na^+ or Cl^- dependent step either apical or basal-lateral Na^+ or Cl^- in the bathing solution was replaced with either a Na^+-free (choline replacement) or a Cl^--free (isethionate replacement) bathing solution. After a 10 minute preincubation in the appropriate test solution, the short circuit current response to 5 x10^{-7}M adrenaline was recorded, 1 minute after adrenaline adddition. The numbers in parentheses are the number of separate determinations of each condition. Errors are expressed as $\pm$ S.E.M.

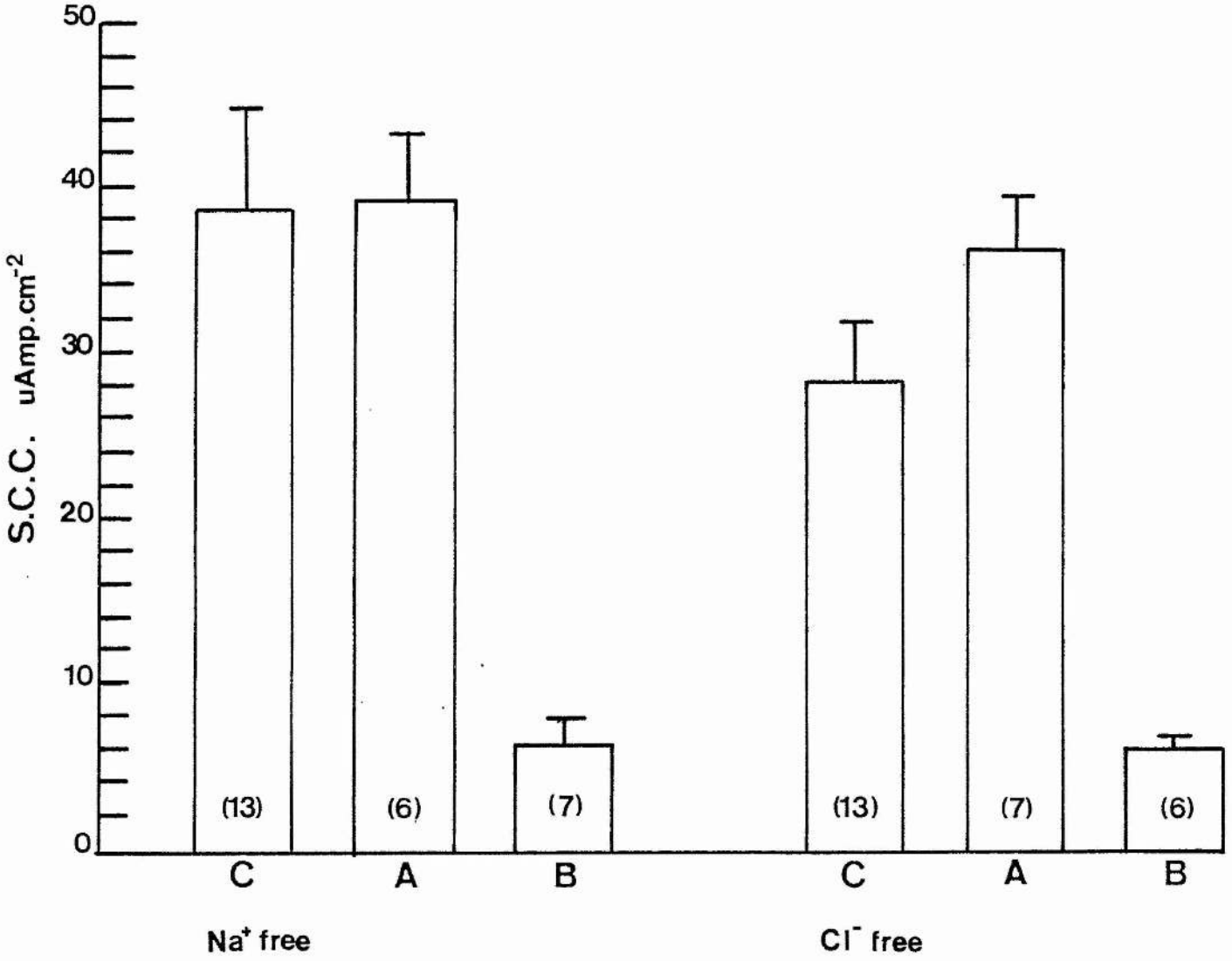

50
40
30
20
10
0
S.C.C. uAmp.cm-2
(13)
(6)
(7)
(13)
(7)
(6)
C
A
B
C
A
B
Na+ free
Cl- free

Fig 3.7 The Cl^- dependence of the adrenaline stimulated short circuit current response.

Ordinate: short circuit current ($uAmps.cm^{-2}$). The effect of chloride free bathing solutions upon the short circuit current response to 2 $x10^{-7}$M adrenaline was tested by preincubating the cells for 10 minutes in the appropriate Cl^--media (apical and basal-lateral Cl^- replacement). The short circuit current was recorded 1 minute after adrenaline addition. The figures in parentheses are the number of separate observations of each condition. Errors are expressed as $\pm$ S.E.M.

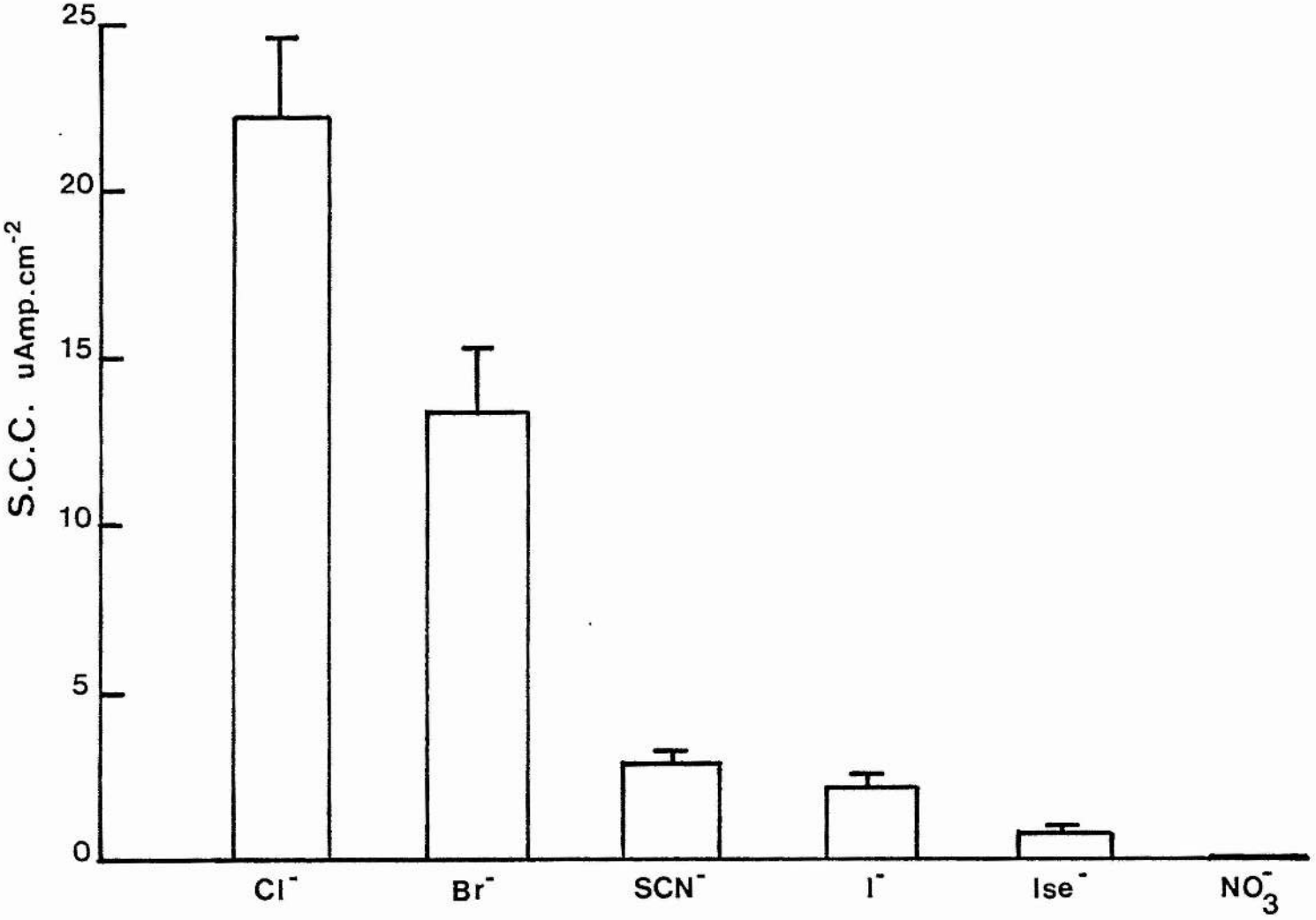

S.C.C. uAmp.cm-2
25
20
15
10
5
0
Cl-
Br-
SCN-
I-
Ise-
NO3-

Fig 3.8 The effect of varying external Cl^- upon the adrenaline stimulated short circuit current.

Abscissa: media Cl^- concentration (mM). Ordinate: short circuit current ($uAmps.cm^{-2}$). Cl^- in the bathing media was varied over the range 0-165 mM by isosmotic replacement of Cl^- by NO_3^-. Cell monolayers were pre-incubated in the appropriate Cl^- concentration for 10 minutes (apical and basal-lateral replacement) . The short circuit current response to 5×10^{-7}M adrenaline was recorded 1 minute after adrenaline addition. The results are the mean $\pm$ S.E.M. of at least 5 separate determinations.

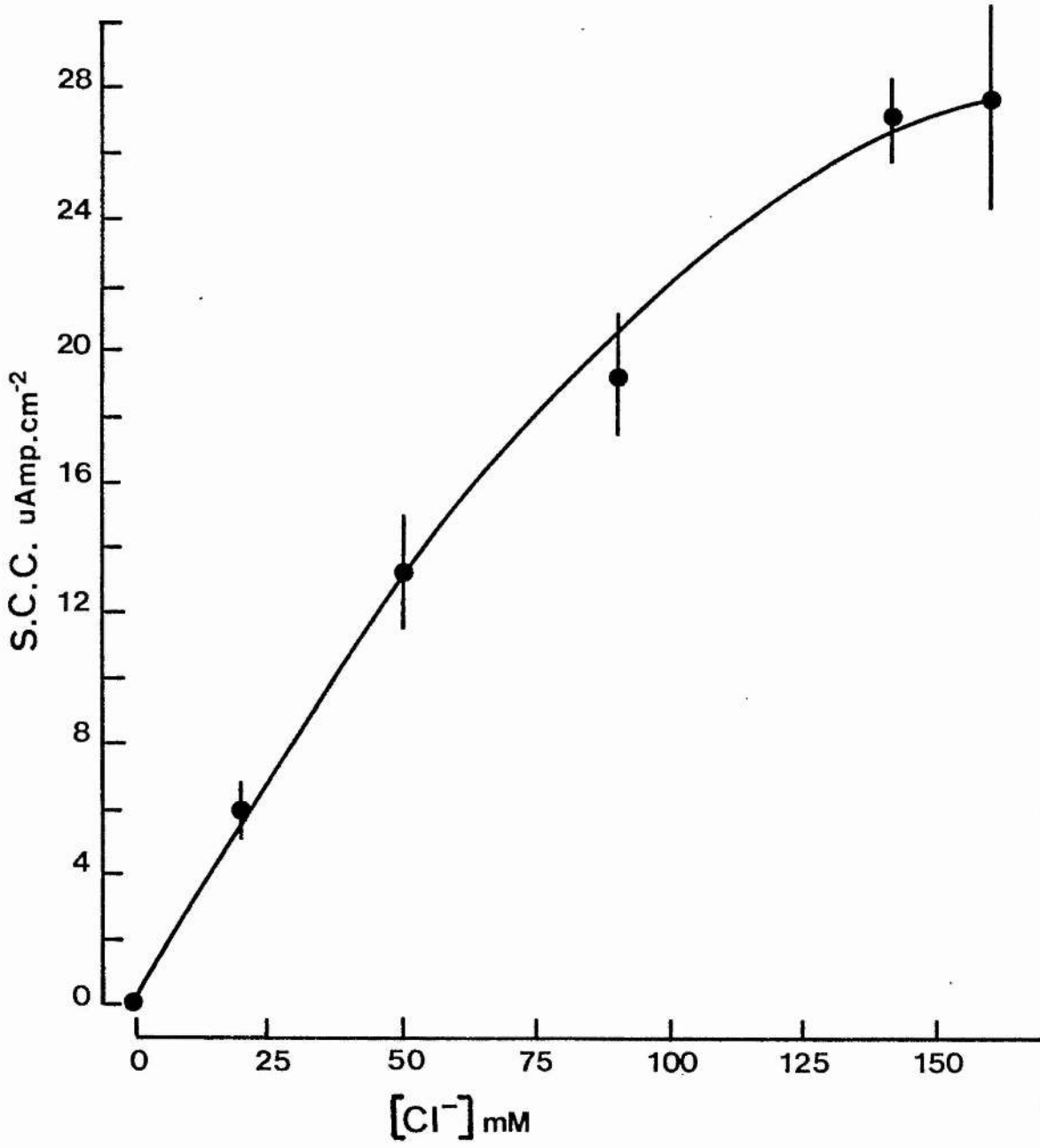

28
24
20
16
12
8
4
0
S.C.C. uAmp.cm-2
0
25
50
75
100
125
150
[Cl-] mM

Fig 3.9 Log dose response curve for adrenaline.

Abscissa: $\log_{10}$ adrenaline concentration. Ordinate: short circuit current (uAmps.cm^{-2}). Log dose response curve for the ability of adrenaline to stimulate short circuit current. The results are the grouped data from 30 monolayers. The concentration of adrenaline tested upon any one monolayer was randomised to avoid systematic error. The short circuit current was recorded 1 minute after adrenaline addition. Figures in parentheses are the number of observations at each adrenaline concentration. Errors are expressed as $\pm$ S.E.M. The half maximal stimulation of short circuit current was at $3.1 \pm 0.3 \times 10^{-8}$M adrenaline, as determined by a Probit analysis of the log dose response curve. A Hill plot of this data gave a similar value.

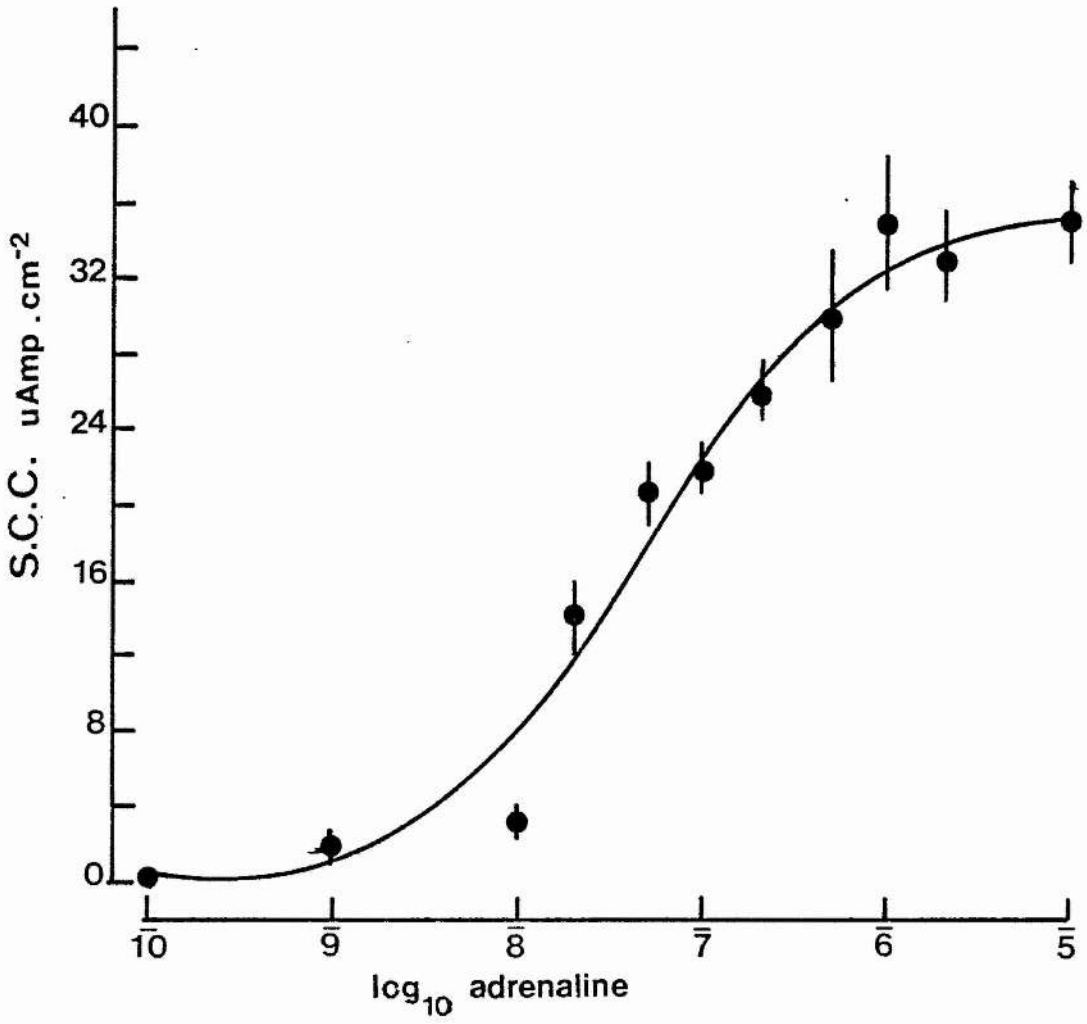

S.C.C. uAmp.cm-2
40
32
24
16
8
0
10̄
9̄
8̄
7̄
6̄
5̄
log10 adrenaline

Fig 3.10 Log dose response curve for isoprenaline.

Abscissa: $\log_{10}$ isoprenaline concentration. Ordinate: short circuit current (uAmps.cm^{-2}). Log dose response curve for the ability of isoprenaline to stimulate short circuit current. The results are the grouped data from 12 monolayers. The concentration of isoprenaline tested upon any one monolayer was randomised to avoid systematic error. The short circuit current was recorded 1 minute after isoprenaline addition. Each data point is the mean $\pm$ S.E.M. of 4 separate determinations. The half maximal stimulation of short circuit current was at $1.5 \pm 0.2 \times 10^{-8}$M, as determined from a Probit analysis of the log dose response curve. A Hill plot of the data presented above gave a similar value.

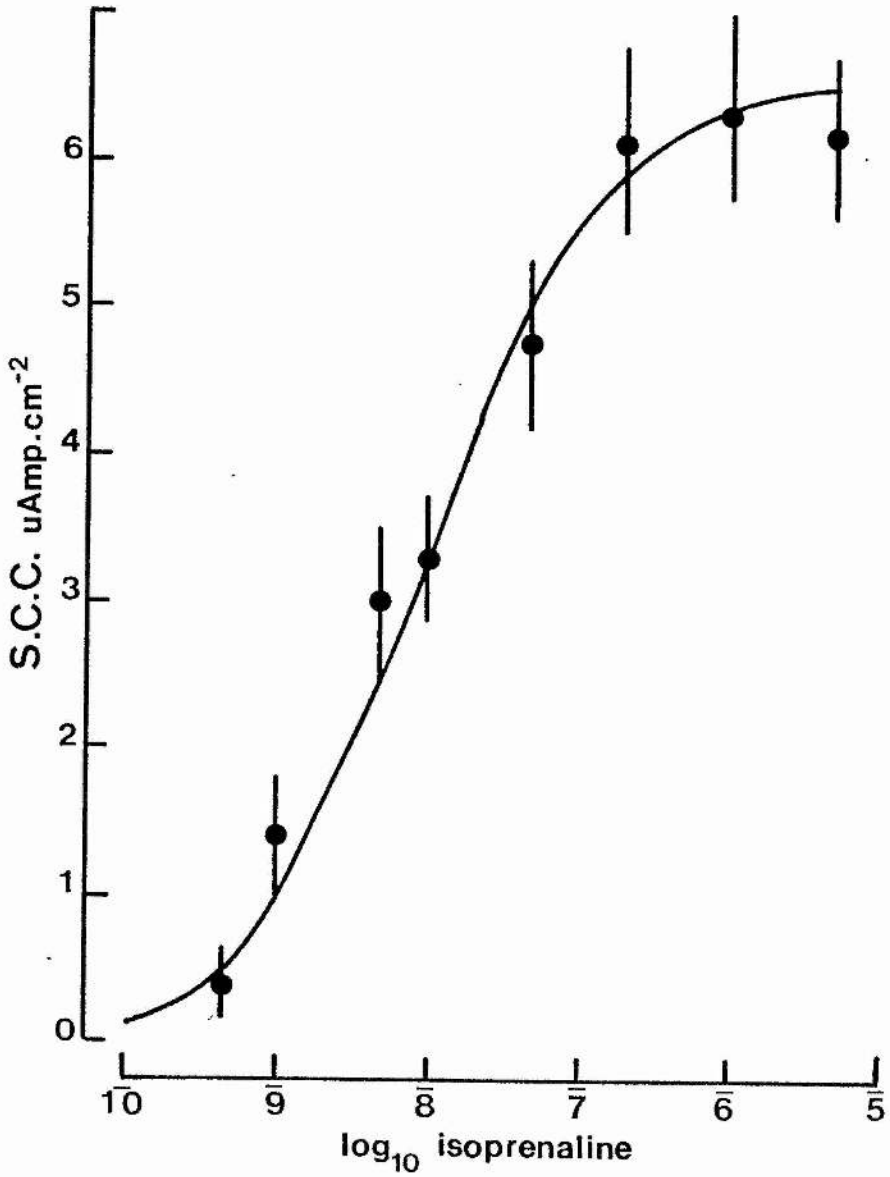

S.C.C. uAmp.cm-2
0
1
2
3
4
5
6
10
9
8
7
6
5
log10 isoprenaline

Fig 3.11 Log dose response curve for noradrenaline.

Abscissa: $\log_{10}$ noradrenaline concentration. Ordinate: short circuit current (uAmps.cm^{-2}). Log dose response curve for the ability of noradrenaline to stimulate short circuit current. The results are the grouped data from 12 monolayers. The concentration of noradrenaline tested upon any one monolayer was randomised to avoid systematic error. The short circuit current response was recorded 1 minute after noradrenaline addition. Each data point is the mean $\pm$ S.E.M. of 4 separate determinations. The half maximal stimulation of short circuit current, dtermined from a Probit analysis, was 6.0 $\pm$ 0.2 x10^{-6}M. A Hill plot gave a similar value.

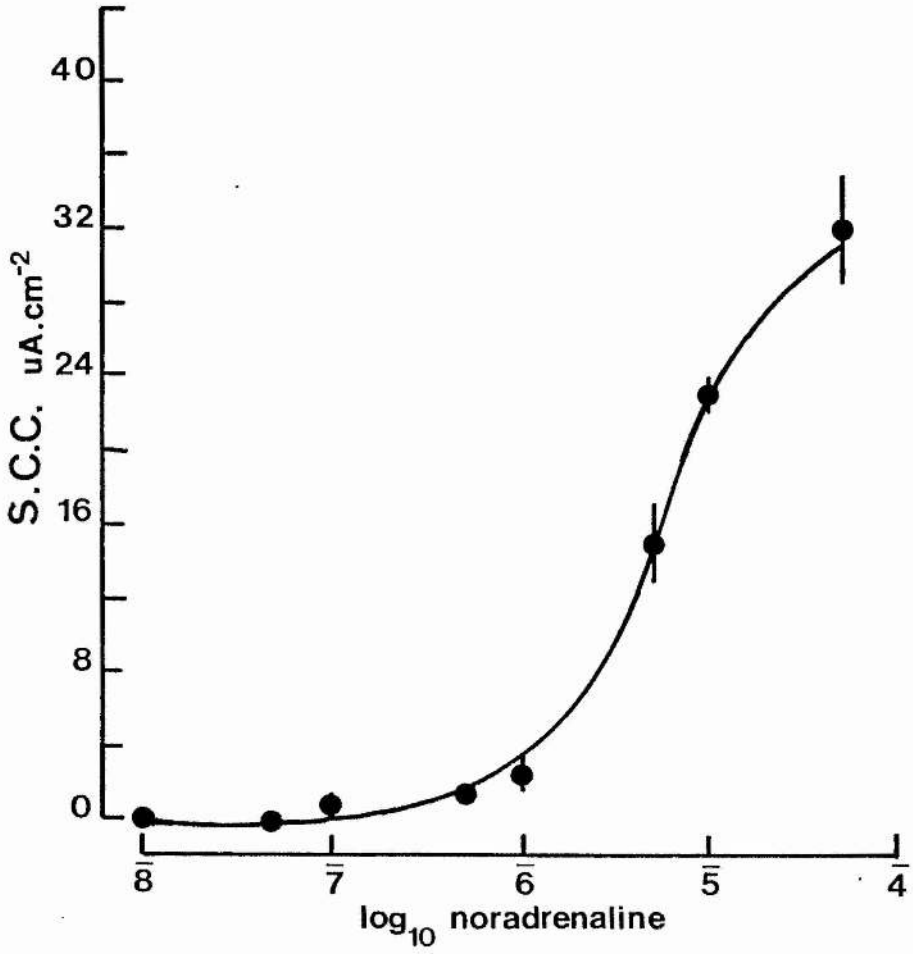

40
32
24
16
8
0
S.C.C. uA.cm-2
8
7
6
5
4
log10 noradrenaline

Fig 3.12 Effect of alpha- and beta-receptor blockade upon the adrenaline stimulated short circuit current response.

Abscissa: time (minutes). Ordinate: short circuit current (uAmps.cm^{-2}). To characterise the receptors involved in adrenaline stimulated short circuit current the sensitivity of the short circuit current to either alpha-receptor blockade, with 2 uM phentolamine, or to beta-receptor blockade with luM propranolol was investigated. Application of propranolol (PR) to the basal-lateral bathing solution during the maintained phase of the adrenaline response led to a rapid decrease in short circuit current. A significant proportion of the response was however proporanolol insensitive. Addition of phentolamine (PH) at this point inhibited the remainder of the propranolol-insensitive, adrenaline stimulated short circuit current. The data is from a single representative experimental trace.

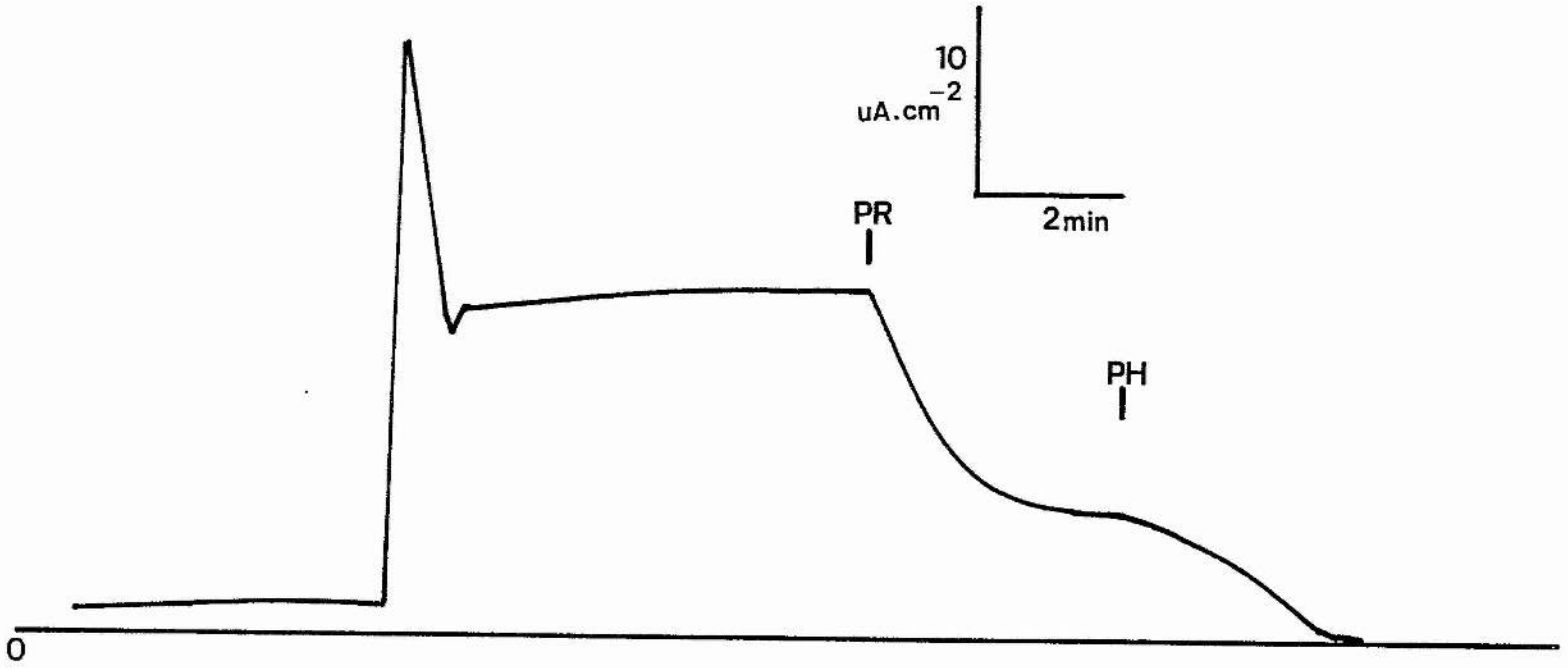
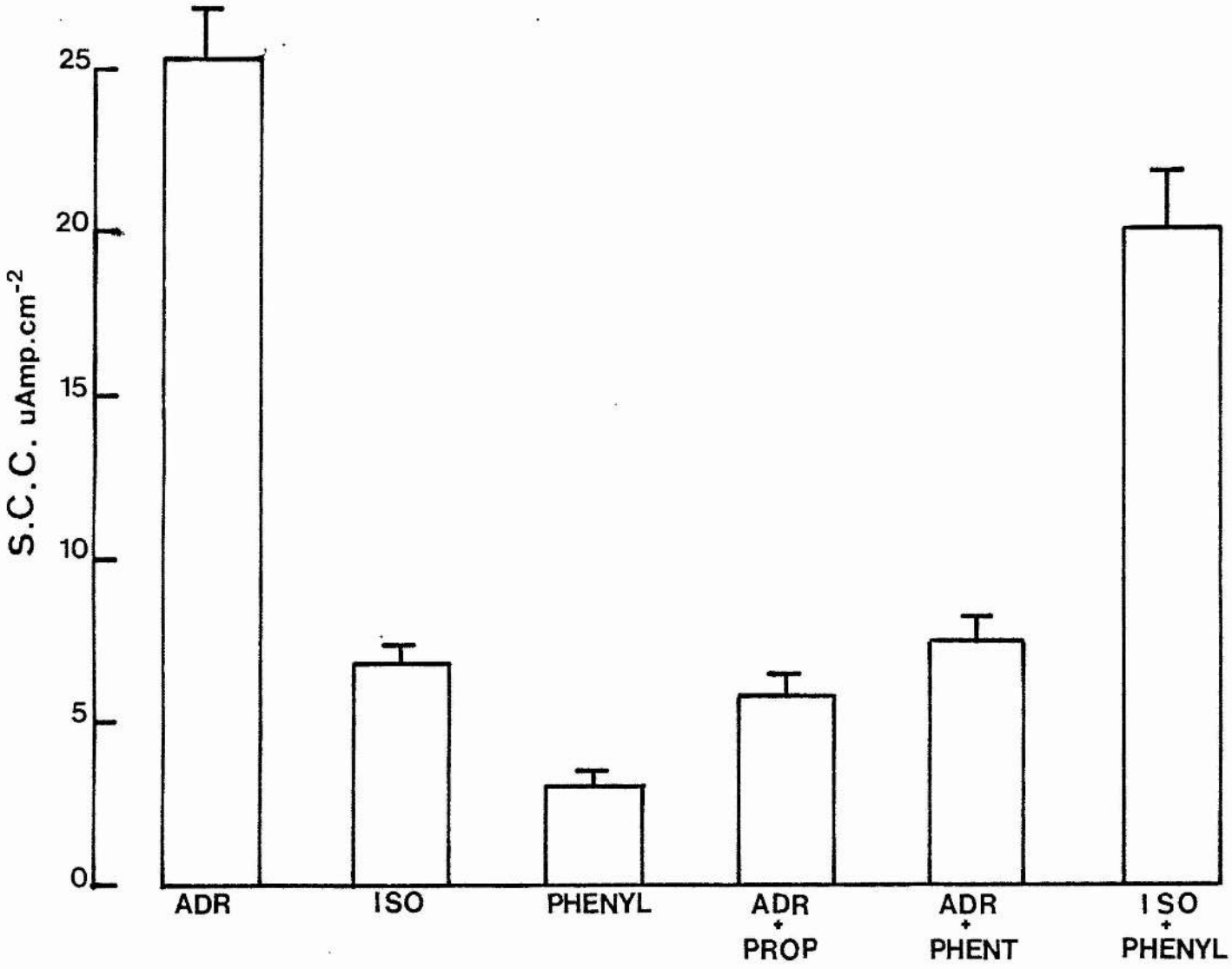

10
uA.cm^{-2}
2min
PR
PH
0

Fig 3.13 The relative contributions of alpha- and beta-receptor stimulated short circuit current to the total adrenaline stimulated short circuit current response.

Ordinate: short circuit current (uAmps.cm^{-2}). A comparison of the ability of adrenaline (5 x 10^{-7}M), isoprenaline (5 x 10^{-7}M), phenylephrine (1 x10^{-4}M) and of isoprenaline (5 x10^{-7}M) + phenylephrine (1 x 10^{-4}M) to stimulate short circuit current. Also shown is the ability of phentolamine (5 uM) or propranolol (5 uM) to inhibit the short circuit current response to 5 x10^{-7}M adrenaline. Propranolol and phentolamine were added 5 minutes before adrenaline. In all cases the short circuit current was recorded 1 minute after agonist addition. The results are expressed as mean $\pm$ S.E.M. of 6 separate determinations of each condition.


S.C.C. uAmp.cm-2
25
20
15
10
5
0
ADR
ISO
PHENYL
ADR + PROP
ADR + PHENT
ISO + PHENYL

CHAPTER 4

A COMPARISON OF THE ACTIONS OF LOOP DIURETICS UPON K^+ FLUXES AND ADRENALINE STIMULATED Cl^- SECRETION

Introduction

It has been clear for a number of years that the diuretic furosemide, and latterly the related diuretics bumetamide and piretanide, are effective inhibitors of 'active' Cl^- transport in both Cl^- absorptive (Burg et al, 1973; Humphreys, 1976; Imai, 1977) and Cl^- secretory epithelia (Candia, 1973; Candia & Schoen, 1978; Frizzell & Heintze, 1979; Widdicombe & Welsh, 1980). More recently evidence has accumulated to suggest that the site of action of these drugs is at the site of the Cl^- entry step: that is the lumenal or apical membrane of absorptive tissues (Burg, 1976; Humphreys, 1976), and the contra-luminal or basal-lateral cell membrane of secretory epithelia (Davis et al, 1977; Eveloff et al, 1978; Frizzell et al, 1979), although in corneal (Candia et al, 1981) and ciliary body epithelium (Saito et al, 1980) the site of action is not clear. In the light of the current mechanistic models of transepithelial Cl^- transport (discussed in detail in Chapter 3, but see Field, 1978; Frizzell et al, 1979; Frizzell & Duffey, 1980) it is possible that the loop diuretic sensitive Cl^- uptake step across either the apical or basal-lateral cell membrane is mediated by an identical electroneutral coupled Na + Cl transport mechanism.

A possible candidate for this coupled electroneutral Na + Cl uptake mechanism is the loop diuretic sensitive $Na^+ + K^+ + Cl^-$ cotransport system which has now been identified in a wide variety of diverse tissues (Bakker-Grunwald, 1981; Chipperfield, 1980; Geck et al, 1980; Aull, 1981; Aiton et al, 1981b; Greger & Schlatter, 1981). In Cl^- absorptive epithelia the cotransport system has been identified to be present upon the apical cell membrane (Eveloff et al, 1980; Greger &

Schlatter, 1981; Oberleitner & Giebisch, 1981) and upon the basal-lateral cell membrane of Cl^- secretory epithelia (Eveloff et al, 1978) in agreement with physiological site of action of the loop diuretics in these tissues (see above).

The $Na^+ + K^+ + Cl^-$ cotransport system is characterised by: (1) A stimulation of Na^+ influx by both K^+ and Cl^- in the external bathing media. Similarly K^+ influx is stimulated by Na^+ and Cl^-, and Cl^- influx is stimulated by Na^+ and K^+. These components of influx were insensitive to inhibition by ouabain but were strongly inhibitable by the loop diuretics; furosemide, bumetamide and piretanide (Bakker-Grunwald, 1978, 1981; Dunham et al, 1980; Greger & Schlatter, 1981). Analysis of the fluxes shows that the dependence of the diuretic sensitive flux of one ion upon a second is saturable and that a tight coupling exists between the fluxes of Na^+, K^+ and Cl^-, with an apparent stoichiometry of 1 Na^+ : 1 K^+ : 2 Cl^- (Geck et al, 1980; Greger & Schlatter, 1981; McRoberts et al, 1982). (2) The cotransport exhibits a high specificity for Na^+, K^+ and Cl^-: Na^+ could be replaced by Li^+ but not by choline, K^+ could be replaced only by Rb^+, and Cl^- could be replaced by Br^- but not by I^-, NO_3^-, SCN^- or isethiocynate (Geck et al, 1980; Chipperfield, 1981; Aiton et al, 1982; Rindler et al, 1982; McRoberts et al, 1982). This is in contrast to the less selective nature of an ion channel or of a coupled Na^+/H^+ and Cl^-/OH^- double exchange (Turnberg et al, 1970; Liedtke & Hopfer, 1982a, 1982b). (3) Since ion fluxes through the diuretic sensitive system are not affected by changes in membrane potential, and complete inhibition of the system has no effect upon membrane potential, the cotransport system is thought to be electroneutral, consistent with proposed stoichiometry of 1 Na^+ : 1 K^+ : 2 Cl^- (Geck

et al, 1980; McRoberts et al, 1982). (4) $Na^+ + K^+ + Cl^-$ cotransport is ouabain, amiloride and SITS insensitive. SITS insensitivity, coupled with the lack of effect of imposing a pH gradient across the cell, delineated the cotransport system from a coupled Na^+/H^+ and Cl^-/OH^- exchange system. The cotransport system is sensitive to inhibition by the loop diuretics; furosemide, bumetamide and piretanide. Although there is some variation in the absolute potency of these agents in different tissues, a potency series of bumetamide $>$ piretanide $\gg$ furosemide is usually seen (Geck et al, 1981; Rindler et al, 1982). Phloretin and ethacrynic acid are also effective inhibitors of flux through the cotransport system.

Although there is strong evidence to link the $Na^+ + K^+ + Cl^-$ cotransport system to Cl^- transport in epithelia (see above), the physiological function of the cotransport system is unclear in non-epithelial cells. One proposed possible function for the diuretic sensitive cotransport system is in the regulation of cell volume (fig 4.1) where the cotransport system is thought to result in a gain in cell volume which is opposed by the Na^+-pump (Geck et al, 1980, 1981). The exact mechanism by which this occurs is as yet unclear and is currently the subject of investigation.

Since the MDCK cell-line has been shown to possess the $Na^+ + K^+ + Cl^-$ cotransport system (Aiton et al, 1981b; Rindler et al, 1982; McRoberts et al, 1982), and since the cotransport system has been implicated in the control of epithelial Cl^- transport in a number of epithelia (see above), it was decided to compare the actions of the loop diuretics upon the cotransport system, using ouabain insensitive K^+ influx as an assay of cotransport activity, with the effects of these agents upon

adrenaline stimulated Cl^- secretion. The results presented in this chapter demonstrate striking correspondence between the properties of the cotransport system and adrenaline stimulated Cl^- secretion, suggesting that $Na^+ + K^+ + Cl^-$ cotransport plays a central role in epithelial Cl^- secretion in MDCK cell epithelia.

RESULTS

(A) Adrenaline-stimulated Cl^- Secretion

(i) The Actions of Adrenaline and Loop Diuretics upon Cl^- Fluxes Across MDCK Cell Monolayers Free from Edge Damage.

In the previous chapter the actions of adrenaline upon net Na^+, K^+ and Cl^- fluxes across MDCK cell monolayers were described. The results suggest that adrenaline stimulates a net electrogenic Cl^- secretion from the basal-lateral to the apical cell surface but has no effect upon the net movements of either Na^+ or K^+. In those experiments, since a second suitable isotope was not available, the uni-directional Cl fluxes; J_{a-b} and J_{b-a} were measured sequentially using a single isotope technique (see methods section for fuller description), a protocol that is inherently less accurate than measuring bi-directional fluxes simultaneously using a double isotope technique. With the availability of a second tracer for Cl^- and the development of a protocol for mounting large area MDCK cell monolayers without appreciable crush damage, procedures that significantly improve the accuracy of trans-epithelial Cl^- flux measurements, it was decided to remeasure bi-directional Cl^- fluxes under control and adrenaline stimulated conditions and to extend the measurements to include an investigation of the effects of the loop diuretics; furosemide, bumetamide and piretanide, upon the adrenaline stimulated Cl^- flux.

Table 4.1 summarises the results of these experiments. Under control conditions there was no significant net flux of Cl^-, in agreement with previous results and the small magnitude of the short circuit current measured over the flux period. A comparison of the uni-directional Cl^-

permeabilities for edge damage free monolayers PCl^-_{a-b} and PCl^-_{b-a} were 2 and 1.7 $cm.hr^{-1}(x10^3)$ respectively, compared with 5.1 and 4.6 $cm.hr^{-1}$ $(x10^3)$ for 1.76 cm^2 area monolayers, suggests that Cl^- exchange through the paracellular shunt is significantly reduced when monolayers are mounted without edge damage.

Addition of 2uM adrenaline to the basal-lateral bathing solution stimulates a significant net Cl^- secretion from the basal-lateral to the apical cell surface (Table 4.1). The magnitude of the net Cl^- flux was sufficient to account for the total increase in short circuit current over the flux measurement period (Table 4.1). The adrenaline stimulated Cl^- secretion was primarily the result of an increased basal-lateral to apical Cl^-. Adrenaline had no significant effect upon the apical to basal-lateral Cl^- flux. Apart from the magnitude of the bi-directional Cl^- fluxes these results confirm and validate the data presented in the previous chapter.

Table 4.1 also summarises the effects of the loop diuretics; furosemide, bumetamide and piretanide upon adrenaline stimulated bi-directional Cl^- fluxes. In the presence of $1x10^{-4}M$ furosemide over 85% of the adrenaline stimulated net Cl^- secretion was inhibited. Similar results were obtained with $1x10^{-4}M$ bumetamide (61%) and $1x10^{-4}M$ piretanide (61%). The loop diuretics acted by blocking the adrenaline stimulated increase in basal-lateral to apical Cl^- flux. They had no significant effect upon the magnitude of the apical to basal-lateral Cl^- flux. A significant proportion of the adrenaline stimulated Cl^- secretion was loop diuretic-insensitive ($p < 0.01$ for bumetamide and $p < 0.05$ for piretanide. See also figs 4.3, 4.4 and 4.5).

The loop diuretics have a basal-lateral site of action (fig 4.2). Apical

application had no inhibitory effect upon adrenaline stimulated Cl^- secretion (see also Table 3.3). Inhibition of JCl_{b-a} by the loop diuretics is consistent with the observation (Table 3.2) that furosemide inhibits the electrogenic component of Cl^- secretion but has no effect upon the adrenaline dependent increase in apical membrane conductance.

(ii) Log Dose Response Curves for the Actions of the Loop Diuretics upon Cl^- Secretion.

Log_{10} dose response curves for the abilities of furosemide, bumetamide and piretanide to inhibit adrenaline stimulated short circuit current (Cl^- secretion) are shown in figs 4.3, 4.4 and 4.5. The action of furosemide upon Cl^- secretion was of high affinity. Half maximal inhibition of the response was observed at $2.5 \pm 0.4 \times 10^{-6}$M. A Hill plot of the log dose response curve (fig 4.3) had a slope close to one, consistent with the interaction of one furosemide molecule with one receptor site (Saito et al, 1980). Similar results were obtained for both bumetamide, apparent K_i $0.8 \pm 0.1 \times 10^{-6}$M (fig 4.4), and piretanide, apparent K_i $4.8 \pm 0.3 \times 10^{-6}$M (fig 4.5).

(B) A Summary of K^+ Influx across MDCK Cell Monolayers.

(i) K^+ Influx across the Apical and Basal-lateral Cell Membranes of High Resistance MDCK cells.

Fig 4.6 shows that K^+ influx into confluent cell monolayers of MDCK cells consists primarily of flux across the basal-lateral cell membrane, flux across the apical cell membrane accounting for less than 1% of the total flux. K^+ influx across the basal-lateral cell membrane is composed of three major components: A ouabain sensitive component of flux - mediated by the $(Na^+ + K^+)$-ATPase and responsible for active K^+ accumulation by

the cell. In the presence of ouabain a significant passive K^+ influx remained (fig 4.6 and 4.7). A significant proportion of this passive influx was sensitive to inhibition by the loop diuretics; furosemide, bumetamide and piretanide (fig 4.6 and 4.7; Aiton et al, 1981a, 1981b; Rindler, 1982). The remainder of the K^+ influx (fig 4.6) was insensitive to inhibition by ouabain plus furosemide and is thought to represent the ground permeability of the membranes to K^+. In contrast to K^+ influx across the basal-lateral cell membrane, influx across the apical membrane consisted of one component and was insensitive to inhibition by either ouabain or ouabain plus furosemide. That ouabain and furosemide were only effective inhibitors of K^+ influx across the basal-lateral cell membranes when added to the basal-lateral bathing solution is consistent with (1) the autoradiographic (Lamb et al, 1981), immunofluorescent (Louvard, 1980) and physiological (Cereijido et al, 1980a; Simmons, 1981a, 1981d) localisation of the $(Na^+ + K^+)$-ATPase to the basal-lateral cell aspects, and (2) with the site of action of furosemide upon the adrenaline stimulated short circuit current (fig 4.2).

Since (a) 99% of the total K^+ influx into MDCK cells consists of flux across the basal-lateral cell border and (b) only flux across the basal-lateral cell membrane consists of a ouabain-sensitive and furosemide sensitive component of flux, measurement of total influx (across both cell borders) into subconfluent cell monolayers will primarily reflect K^+ flux across the basal-lateral cell membrane and so provide an alternative experimental preparation with which to investigate the three components of K^+ fluxes across the basal-lateral cell border. Fig 4.7 shows that the magnitude of the total influx into subconfluent cell

monolayers was similar to that into confluent monolayers grown upon filter supports (compare fig 4.2 and fig 4.7). Fig 4.7 also shows that influx into subconfluent cell monolayers was composed of a ouabain sensitive flux, a furosemide sensitive flux, and a ouabain and furosemide flux. The three flux components were roughly additive, suggesting that ouabain and furosemide had different sites of action (e.g. furosemide does not affect pump flux). This conclusion is supported by the observations of Karlish and colleagues that in proteoliposomes containing purified Na^+ - pumps addition of the loop diuretic furosemide had no effect upon ^{86}Rb (K^+) influx. Fig 4.8 shows that a similar proportion of the ouabain insensitive K^+ influx was sensitive to inhibition by the related loop diuretics bumetamide and piretanide, and also that the effects of these diuretics were not additive. After maximum inhibition by one diuretic no further inhibition was seen when a second diuretic was added, implying that these diuretics act upon one site. Table 4.2 shows that replacement of media Cl^- by NO_3 inhibited a similar proportion of the K^+ influx as the loop diuretics; furosemide, bumetamide and piretanide. A pharmacological characterisation of the ouabain insensitive, furosemide sensitive, component of K^+ influx into MDCK cells has recently been published (Aiton et al, 1981b; Aiton et al, 1982; Rindler et al, 1982) and is outwith the scope of this study.

(ii) Dose Dependency of the Diuretic Sensitive Component of K^+ Influx.

Fig 4.9 shows a $\log_{10}$ dose response curve for the ability of furosemide to inhibit the ouabain insensitive component of K^+ influx. Maximal inhibition of this component was seen with $1x10^{-4}M$ furosemide and half maximal inhibition of the ouabain insensitive K^+ influx was obtained with $1.6 \pm 0.2 \times 10^{-6}M$ furosemide. A Hill plot of this data revealed a

slope of unity consistent with a 1:1 interaction between drug and receptor. Similar results were obtained for bumetamide, K_i = 3.8 ± 0.1 x10^{-7}M (fig 4.10), and piretanide, K_i = 1.6 ± 0.4 x10^{-6}M (fig 4.11).

(iii) A Comparison of the Time Course of Furosemide Action upon K^+ Influx and Cl^- Secretion.

Fig 4.12 shows the time course of inhibition of K^+ influx by furosemide. It is clear from this figure that furosemide action is rapid; maximum inhibition of the ouabain sensitive component of K^+ influx is achieved within the first five minutes of incubation. No further inhibition of influx is observed after prelonged exposure to furosemide. The time course of inhibition of Cl^- secretion by furosemide is similar (fig 4.2).

(C) A Summary of K^+ Efflux across MDCK Cell Monolayers.

Fig 4.13 shows the fractional loss of K^+ across the apical and basal-lateral cell membranes of MDCK cell monolayers grown upon permeable filter supports. Although fractional efflux across the basal-lateral cell membranes was time dependent for the first six minutes, indicating a contribution of loss of isotope from the millipore filter space (see Chapter 5 for an analysis of K^+ efflux kinetics), the basic pattern of a relatively impermeable apical membrane coupled to a permeable basal-lateral cell membrane is still evident. That K^+ loss across the basal cell border was only 25-fold greater than K^+ loss across the apical cell border in comparison to the 100-fold difference in apical to basal-lateral K^+ influx, suggests that there should be a net flux of K^+ across the apical border under steady-state conditions. Since this is clearly not the case (see Table 3.1) other possible explanations must be considered, the most likely being that due to their small magnitude

estimation of apical influx and efflux are subject to some error. A second possibility is that some loss of K^+ occurs across the epithelium via a paracellular pathway from the basal-lateral to apical solutions (but see Chapter 5).

Fig 4.14 shows the effects of various experimental manoeuvres upon efflux from subconfluent MDCK cell monolayers. Addition of $1x10^{-4}M$ furosemide to the bathing media resulted in a prompt decrease in fractional K^+ to about 60% of the control value. Ouabain plus furosemide had a similar inhibitory effect upon fractional K^+ loss. In contrast to its inhibitory action upon K^+ influx, addition of $1x10^{-3}M$ ouabain stimulated a time-dependent increase in fractional K^+ loss which was diuretic sensitive. This can be accounted for by a stimulation of K^+: K^+ exchange fluxes by increased intracellular Na^+ levels (J.F. Aiton and N.L. Simmons, unpublished results; Bakker-Grunwald et al, 1982). The actions of these agents upon K^+ influx and K^+ efflux are consistent with the diuretic sensitive component of K^+ flux under control conditions being involved primarily in K^+: K^+ exchange.

Discussion

In the last two years a ouabain insensitive but furosemide sensitive component of K^+ influx into MDCK cells has been reported by several groups. On the basis of extensive pharmacological studies (Aiton et al, 1981b, 1982; Rindler et al, 1982; McRoberts et al, 1982), the diuretic sensitive component of K^+ influx has been shown to exhibit many of the characteristics of a coupled $Na^+ + K^+ + Cl^-$ cotransport system previously identified in a number of other tissues, such as red blood cells and Erlich Ascites tumour cells (for references see introduction). In addition to K^+ a similar component of Na^+ and Cl^- fluxes has also been identified (Rindler et al, 1982; McRoberts et al, 1982).

Since the furosemide sensitive cotransport system may be responsible for Cl^- accumulation within a variety of cells (Chipperfield, 1980, 1981; Geck et al, 1980) including Cl^- secretory epithelia (Eveloff et al, 1978), it was decided to address the question of whether the coupled $Na^+ + K^+ + Cl^-$ cotransport was involved in secretagogue stimulated Cl^- secretion by MDCK cells? To answer this question the effects of loop diuretics upon adrenaline stimulated Cl^- secretion were compared with their effects upon the cotransport system using the ouabain insensitive, diuretic sensitive component of K^+ influx as an assay of cotransport function. K^+ influx was used in preference to Cl^- influx because K^+ influx is easier to measure (Eveloff et al, 1978) whilst giving the same results as measuring Cl^- (Rindler et al, 1982; McRoberts et al, 1982).

The results presented in this study confirm the existence of a loop diuretic sensitive component of K^+ flux in MDCK cell monolayers dependent upon the presence of Cl^- in the external bathing solution, and extend

these observations by demonstrating that (1) K^+ flux across MDCK cell monolayers is dominated by flux across the basal-lateral cell membrane, flux across the apical membrane being of little quantitative importance. (2) The results imply that the diuretic sensitive component of K^+ influx (i.e. the $Na^+ + K^+ + Cl^-$ cotransport system) is located solely upon the basal-lateral cell aspects. This localisation is consistent with the site of action of furosemide in secretory epithelia (see below). In contrast, in thick ascending limb of the loop of Henle cells, the cotransport system is thought to be localised upon the apical cell membrane (Greger, 1981). These two results suggest that the location of the cotransport system is dependent upon the physiological function of the epithelia in which it appears.

Measurement of transepithelial Cl^- fluxes using ^{36}Cl and ^{77}Br as tracers of unidirectional Cl^- movements gave similar results to those presented in Chapter 3: (a) under control conditions there was no significant net Cl^- flux, (b) addition of adrenaline to the basal-lateral bathing solution stimulated a significant secretion from the basal-lateral to the apical bathing solution, and (c) Cl^- secretion was electrogenic (JCl_{net} = SCC) and primarily the result of a stimulation of JCl_{b-a}. The flux measurements also demonstrated that a significant part of the adrenaline stimulated short circuit current (60 - 80%) was sensitive to inhibition by the loop diuretics; furosemide, bumetamide and piretanide, added to the basal-lateral bathing solution.

Despite the possible errors inherent in estimating net flux on the basis of measuring unidirectional fluxes sequentially on the same monolayer with a single isotope, a comparison of the results in this and the previous chapter validates the use of the single isotope technique as an

accurate measure of net Cl^- fluxes under control and adrenaline stimulated conditions. An indication that the new chamber design does in fact reduce edge damage artefacts, apart from the improvement in transepithelial electrical resistance (Chapter 2), is the significant decrease in bi-directional Cl^- exchange fluxes compared to monolayers mounted with edge damage.

There are some striking parallels between the actions of the loop diuretics upon Cl^- secretion and cotransport: For both systems loop diuretics are only effective when added to the basal-lateral bathing solution, implying the localisation of cotransport sites to this membrane, a similar distribution to that of the coupled diuretic sensitive Cl^- accumulation mechanism in secretory epithelia (Eveloff et al, 1978; Frizzell et al, 1979). The loop diuretics have a similar high affinity for the cotransport system and for their actions upon adrenaline stimulated Cl^- secretion (Table 4.3). The potency series for Cl^- secretion: bumetamide $>$ furosemide $\geqslant$ piretanide is identical to that for inhibition of the cotransport system. A similar potency series has been reported for inhibition of the cotransport system in Erlich Ascites tumour cells (Geck et al, 1981). Table 4.4 summarises the pharmacological similarities between Cl^- secretion and $Na^+ + K^+ + Cl^-$ cotransport. As mentioned above both processes are inhibited by the high ceiling diuretics; furosemide, bumetamide and piretanide, the site of action in each case being the basal-lateral cell membrane. Similarly phloretin inhibits a substantial proportion of both the cotransport system and Cl^- secretion when added to the basal-lateral cell aspects. For Cl^- secretion, phloretin is also effective from the apical bathing solution. The stilbene; SITS is ineffective in inhibiting either Cl^-

secretion (Chapter 3) or the cotransport system. SITS is an effective inhibitor of coupled Na^+/H^+ and Cl^-/OH^- exchange in small intestine apical membrane vesicles (Liedtke & Hopfer, 1982a).

Adrenaline stimulated Cl^- secretion is dependent upon the presence of both Na^+ and Cl^- in the basal-lateral bathing solution. A Na^+ or Cl^- free apical bathing solution has no significant effect upon adrenaline stimulated Cl^- secretion (see Chapter 3). Of a wide range of Na^+ and Cl^- ion replacements only Li^+ and Br^- are able to support a significant proportion of the adrenaline stimulated Cl^- secretion under control conditions. The $Na^+ + K^+ + Cl^-$ cotransport also exhibits an identical ion replacement selectivity - only Li^+ and Br^- are acceptable substitutes for Na^+ and Cl^-. Such a narrow ion replacement selectivity is generally considered to be a feature of a coupled transport mechanism. Ion channels tend to accept a wider range of ions (Liedtke & Hopfer, 1982a, 1982b).

The physiological role of the diuretic coupled cotransport system has been the subject of much discussion (Lew & Beauge, 1979). Proposed roles for the cotransport systems include accumulation of solutes into the cell above their electrochemical equilibrium and in the modulation of cell volume.

In this chapter I have investigated the similarities between the diuretic sensitive component of passive K^+ influx across the basal-lateral cell aspects of MDCK cells and adrenaline stimulated Cl^- secretion. The striking correspondence between the characteristics of the two processes provides strong but indirect evidence that the coupled $Na^+ + K^+ + Cl^-$ cotransport system plays a central role in hormone stimulated Cl^- secretion by MDCK cells.

TABLE 4.1

A summary of bi-directional Cl^- fluxes measured with $^{77}Br/^{36}Cl$ as tracers upon 9.62 cm^2 area monolayers mounted without edge damage. Cl^- fluxes were determined under short circuit conditions plus and minus 2uM adrenaline. The effect of the loop diuretics upon the adrenaline stimulated Cl^- secretion was determined in the presence of 10^{-4} M diuretic. J_{a-b} denotes flux from the apical to the basal-lateral cell surface. J_{b-a} denotes the reverse flux. P_{a-b} was determined from the equation $P_{a-b} = J_{a-b} / C_a$ where C_a is the concentration of Cl in the apical bathing solution.

Significantly different from control values:

a = $p < 0.01$

b = $p < 0.05$

c = non significant

Significantly different from J_{net}:

d = non significant

e = $p < 0.1$

Condition	n	J_{a-b} $umol.cm^{-2}.hr^{-1}$	P_{a-b} $cm.hr^{-1} \times 10^{3}$	J_{b-a} $umol.cm^{-2}.hr^{-1}$	P_{b-a} $cm.hr^{-1} \times 10^{3}$	J_{net} $umol.cm^{-2}.hr^{-1}$	Current flux equivalent $umol.cm^{-2}.hr^{-1}$
Control	(6)	0.32 ± 0.11	2.00 ± 0.60	0.27 ± 0.09	1.70 ± 0.60	0.048 ± 0.06	0.014 ± 0.01[d]
Adrenaline	(11)	0.36 ± 0.08[c]	2.20 ± 0.50[c]	0.96 ± 0.22[a]	6.00 ± 0.90[a]	-0.59 ± 0.12[a]	0.36 ± 0.05[a,d]
Adrenaline + Furosemide	(7)	0.19 ± 0.06[c]	1.20 ± 0.40[c]	0.28 ± 0.08[c]	1.76 ± 0.50[c]	-0.09 ± 0.05[c]	0.08 ± 0.13[c,d]
Adrenaline + Bumetamide	(6)	0.22 ± 0.04[c]	1.40 ± 0.28[c]	0.45 ± 0.06[c]	2.81 ± 0.40[c]	-0.23 ± 0.04[a]	0.12 ± 0.03[a,e]
Adrenaline + Piretanide	(3)	0.31 ± 0.08[c]	1.90 ± 0.47[c]	0.53 ± 0.11[c]	3.30 ± 0.60[c]	-0.23 ± 0.10[b]	0.09 ± 0.02[c,d]

TABLE 4.2

The effect of replacing Cl^- by NO_3^- upon ^{86}Rb influx and efflux from subconfluent monolayers of MDCK cells. Influx was measured over a five minute flux period. Efflux is expressed as the ratio of ^{86}Rb loss at T = 2 minutes against the rate of ^{86}Rb loss at T = 0 minutes. Cl^- was replaced by NO_3^- at T = 0 minutes. The results are expressed as means $\pm$ S.E. The numbers in parentheses are the number of observations at each point.

Significantly different
from control values:

a = $p < 0.01$

Condition	K^+ Influx mmol/l.c.w./min^{-1}	K^+ Efflux T_2/T_0
Control (160mM Cl^-)	4.24 ± 0.22 (4)	0.89 ± 0.06 (4)
Cl^- - free (160mM NO_3^-)	2.92 ± 0.3^a (4)	0.69 ± 0.04^a (4)

TABLE 4.3

A comparison of the apparent K_i values for the ability of furosemide, bumetamide and piretanide to inhibit the diuretic sensitive component of K^+ influx and adrenaline stimulated Cl^- secretion. K_i values are determined from a Probit analysis of the data presented in this chapter.

Diuretic	Apparent K_1 K^+ influx	Apparent K_1 Cl^- secretion
Furosemide	1.6 ± 0.2 uM	2.5 ± 0.4 uM
Bumetamide	0.4 ± 0.1 uM	0.8 ± 0.1 uM
Piretanide	1.6 ± 0.4 uM	4.8 ± 0.3 uM

TABLE 4.4

Parallels between the diuretic sensitive cotransport system and the adrenaline stimulated Cl^- secretion in MDCK cell epithelium. Data for the properties of the cotransport system are taken from the following papers: Aiton et al, 1981a, 1981b, 1982; Rindler et al, 1982; McRoberts et al, 1982. Data for the cotransport system and for adrenaline stimulated Cl^- secretion is also taken from the present results.

	$Na^+ + K^+ + Cl^-$ cotransport	Adrenaline stimulated Cl^- secretion
Inhibition by:	✓	✓
Furosemide	✓	✓
Bumetamide	✓	✓
Piretanide	✓	✓
Phloretin	✓	✓
SITS	✗	✗
Effectiveness of Na^+ replacement with:		
Li^+	partial	partial
Choline	No	No
Tris	No	No
Effectiveness of Cl^- replacement with:		
Br^-	partial	partial
NO_3^-	No	No
I^-	No	No

Fig 4.1 The proposed role for the $Na^+ + K^+ + Cl^-$ cotransport system in the regulation of cell volume.

The role of the cotransport system is to counteract the 'shrinking' effect of the $(Na^+ + K^+)$-ATPase by transporting Na^+, K^+ and Cl^- ions into the cell. The cotransport system is under feedback control. It is proposed that as the cell swells under the influence of the cotransport system its volume exceeds a threshold value and the swelling action of the cotransport system is switched off. Experimental evidence has demonstrated that the threshold volume can be modulated with theophylline, although since there are no corresponding changes in cyclic nucleotide levels the mechanism remains unclear. The model of volume regulation shown in fig 4.1 was adapted from that proposed by Geck and colleagues (1980, 1981) for Erlich tumour cells.

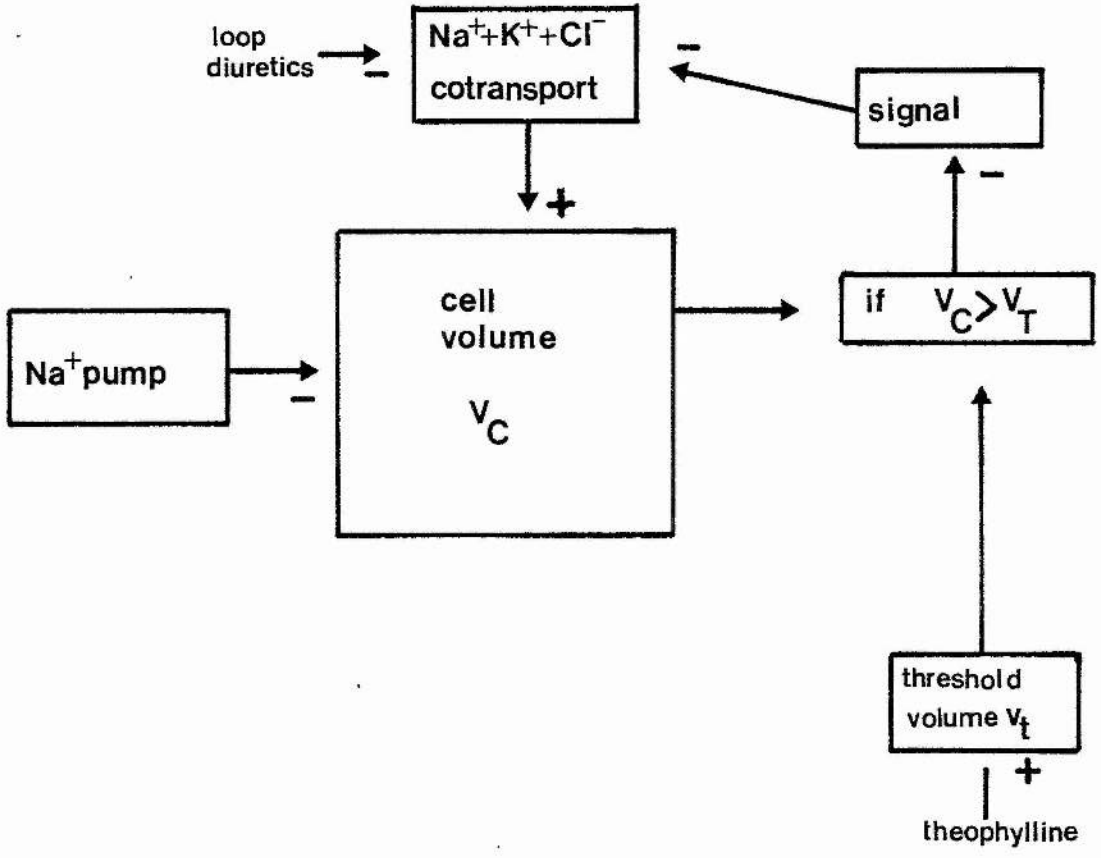

loop
diuretics
$Na^+ + K^+ + Cl^-$
cotransport
signal
cell
volume
V_C
Na^+ pump
if $V_C > V_T$
threshold
volume V_t
theophylline

Fig 4.2 Time-course and site of action for the ability of furosemide to inhibit the adrenaline stimulate short circuit current.

Abscissa: percentage short circuit current. Ordinate: time (minutes). The site of action of furosemide in inhibiting adrenaline stimulated short circuit current was tested by adding 0.1mM furosemide to either the apical (■) or basal-lateral (▲) bathing solution. Furosemide was applied during the maintained phase of the adrenaline stimulated short circuit current (5 minutes after adrenaline addition). Each condition is the mean of 5 separate determinations, error bars are ± SEM. Where error bars are not shown, deviation was always less than 5%.

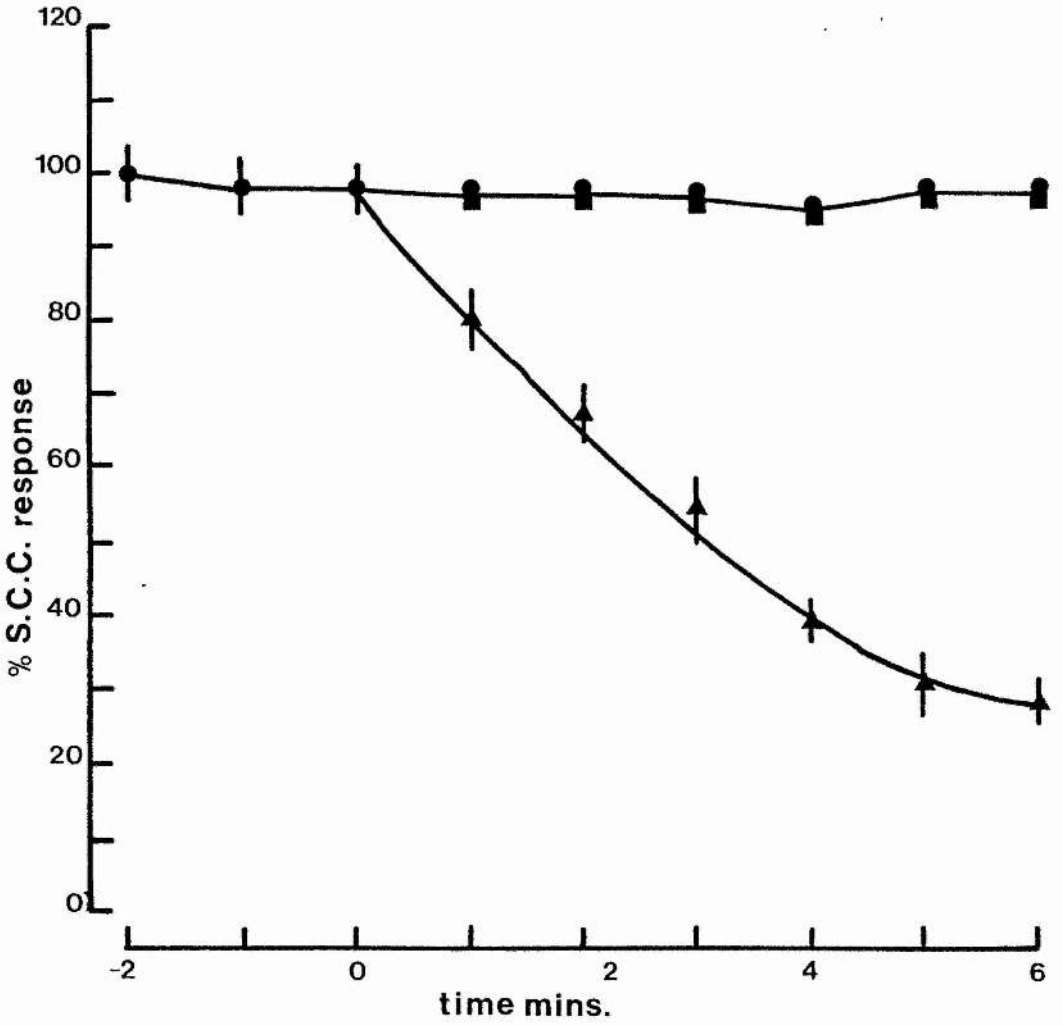

120
100
80
60
40
20
0
% S.C.C. response
-2
0
2
4
6
time mins.

Fig 4.3 Log dose response curve for the ability of furosemide to inhibit the adrenaline stimulated short circuit current response.

Abscissa: percentage of control response to 5 $x10^{-7}$M adrenaline. Ordinate: $\log_{10}$ furosemide concentration. The ability of furosemide to inhibit adrenaline stimulated Cl^- secretion was tested by measuring the inhibitory effects of different concentrations of furosemide upon the maintained phase of the adrenaline stimulated short circuit current (furosemide was added 5 minutes after adrenaline). Half maximal inhibition of the response, calculated from a Probit analysis of the dose response curve, was at 2.5 ± 0.4 $x10^{-6}$M furosemide. The inset shows a Hill plot of the data presented in the log dose response curve. The solid line represents the least-squares regression line, which obeys the equation:

$$y = 1.04(x) + 5.63$$

Both the slope of this line and the correlation coefficient (0.981) were significant ($P < 0.001$). Each datum is the mean $\pm$ SEM of 3 determinations.

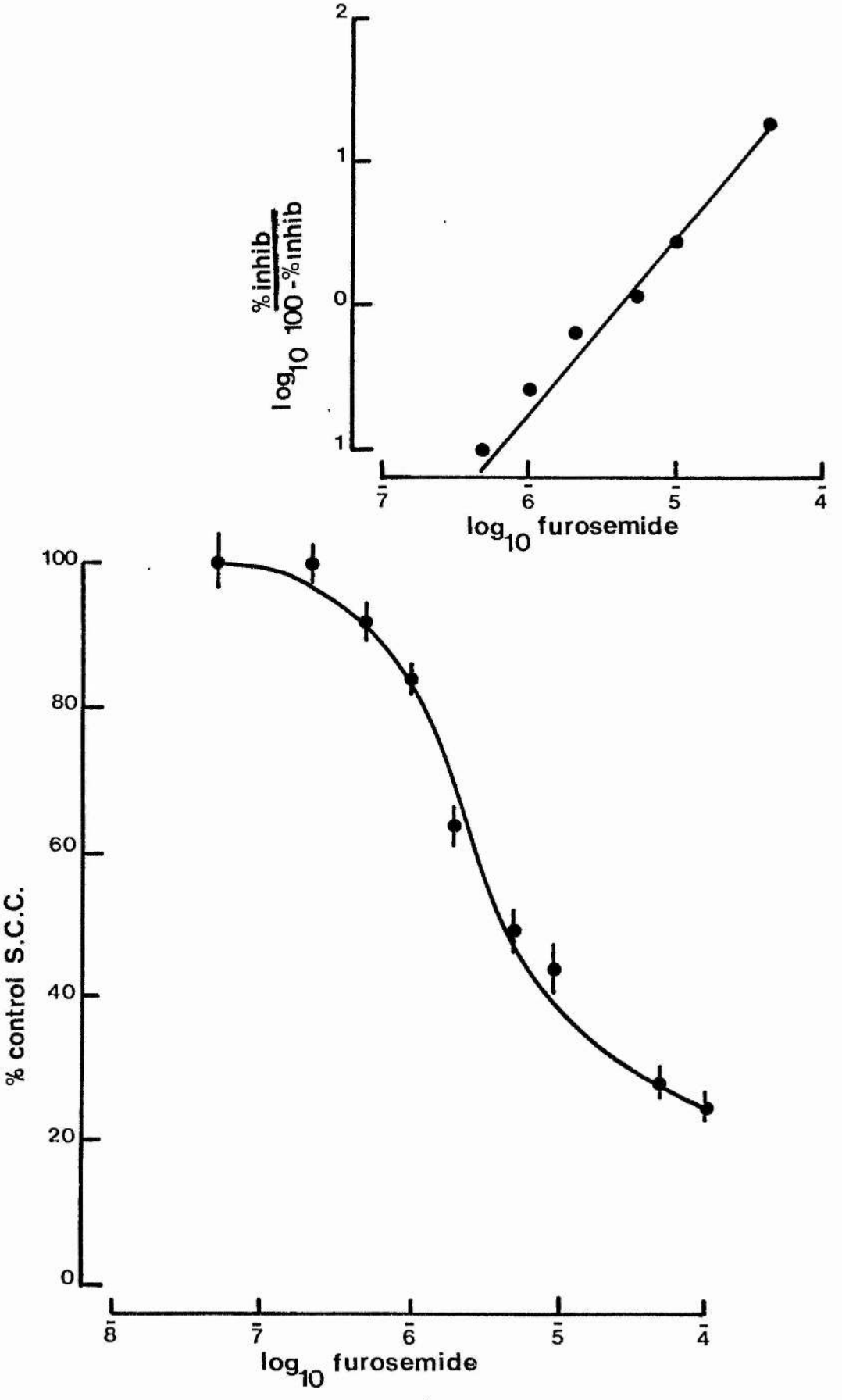

2
1
0
1
$\log_{10} \frac{\%\text{inhib}}{100-\%\text{inhib}}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ furosemide
100
80
60
40
20
0
% control S.C.C.
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ furosemide

Fig 4.4 Log dose response curve for the ability of bumetamide to inhibit the adrenaline stimulated short circuit current response.

Abscissa: percentage of control response to 5×10^{-7}M adrenaline. Ordinate: $\log_{10}$ bumetamide concentration. Inhibition of the adrenaline stimulated short circuit current was measured by adding the appropriate concentration of bumetamide to the basal-lateral bathing solution. Bumetamide was added 5 minutes after adrenaline (the maintained phase of the response). Half maximal inhibition of Cl^- secretion (short circuit current), calculated from a Probit analysis of the log dose response curve, was observed with $0.8 \pm 0.1 \times 10^{-6}$M bumetamide. The inset shows a Hill plot of the dose response curve. The solid line represents the least-squares regression line, which obeys the equation:

$$y = 1.52(x) + 9.52$$

Both the slope and the correlation coefficient for the regression were significant ($P < 0.001$). The correlation coefficient was 0.978. Each datum is a single determination.

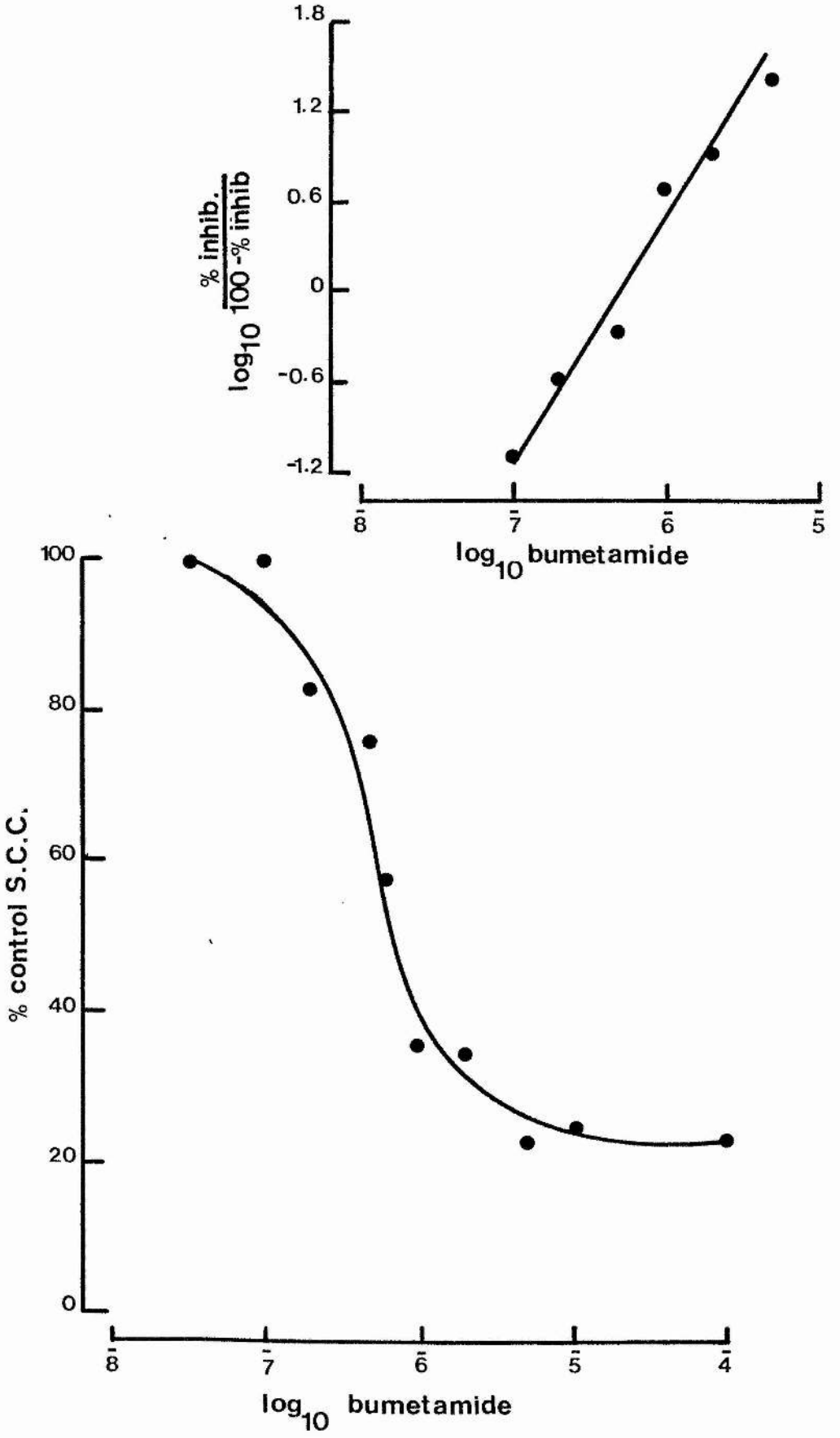

1.8
1.2
0.6
0
-0.6
-1.2
log10 % inhib. / 100 -% inhib
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
log10 bumetamide
100
80
60
40
20
0
% control S.C.C.
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
log10 bumetamide

Fig 4.5 Log dose response curve for the ability of piretanide to inhibit the adrenaline stimulated short circuit current response.

Abscissa: percentage of control response to 5×10^{-7}M adrenaline.

Ordinate: $\log_{10}$ piretanide concentration. Inhibition of the adrenaline stimulated short circuit current by piretanide was measured as the ability of various concentrations of piretanide to inhibit the maintained phase of adrenaline stimulated short circuit current. Piretanide was added to the basal-lateral bathing solution 5 minutes after addition of adrenaline. Half maximal inhibition of short circuit current by piretanide , calculated from a Probit analysis of the log dose response curve, was observed with $4.8 \pm 0.3 \times 10^{-6}$M piretanide. The inset shows a Hill plot of the data presented in the log dose response curve. The solid line represents the least-squares regression line and obeys the equation:

$$y = 1.12(x) + 5.71$$

The slope of the line was significant ($P < 0.001$) and the correlation coefficient was 0.996 ($P < 0.001$). Each datum is a single determination.

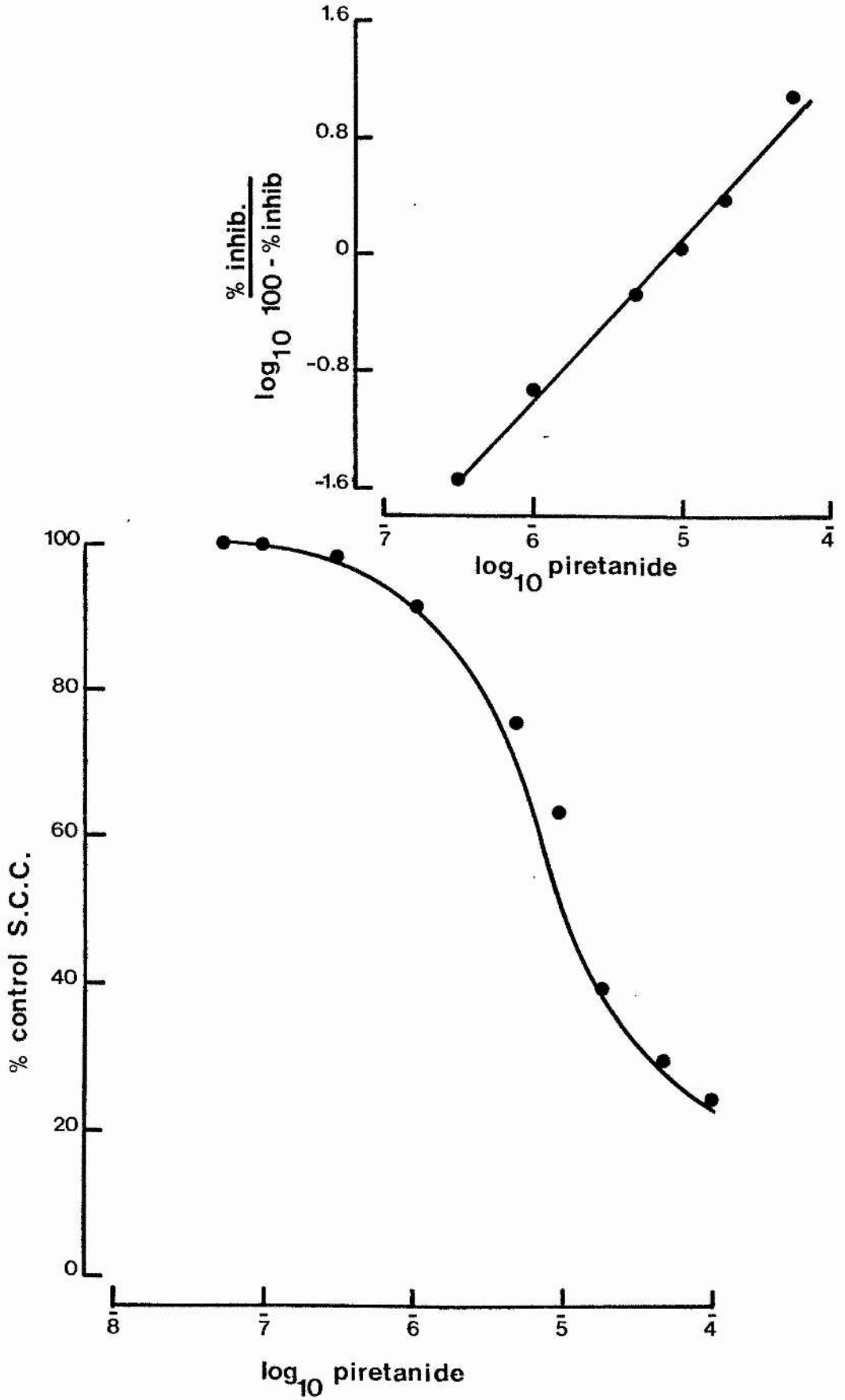

1.6
0.8
0
-0.8
-1.6
log10 % inhib. / 100 - % inhib
7̄
6̄
5̄
4̄
log10 piretanide
100
80
60
40
20
0
% control S.C.C.
8̄
7̄
6̄
5̄
4̄
log10 piretanide

Fig 4.6 The effects of ouabain and ouabain plus furosemide upon K^+ influx across the apical and basal-lateral cell membranes.

A. basal-lateral influx.

Abscissa: ^{86}Rb influx (mmoles/liter cell water/min). K^+ influx across the basal-lateral cell membrane was measured using ^{86}Rb as a tracer for K^+ and a 10 minute flux period. K^+ influx was measured under 3 conditions: control; in the presence of 1mM ouabain and in the presence of 1mM ouabain and 0.1 mM furosemide. The results are expressed as the mean $\pm$ SEM of 6 determinations.

B. apical influx.

Abscissa: ^{86}Rb influx (umoles/liter cell water/min). K^+ influx was measured over a 10 minute influx period. K^+ influx was measured under control conditions, in the presence of 1mM ouabain and in the presence of 1mM ouabain and 0.1mM furosemide. All drugs were applied to the basal-lateral cell surface. The results are expressed as the mean $\pm$ SEM of 6 separate determinations of flux.

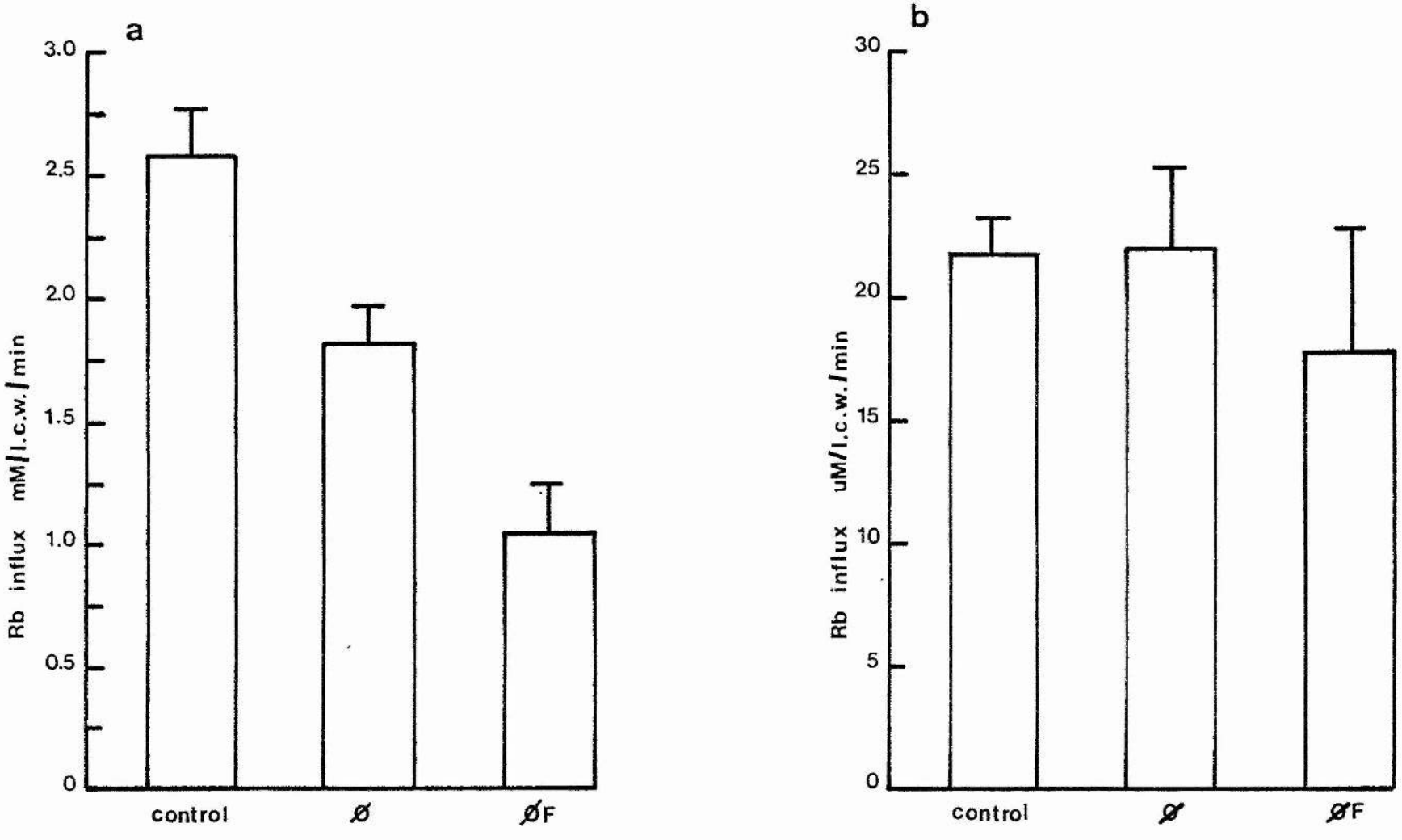

a
3.0
2.5
2.0
1.5
1.0
0.5
0
Rb influx mM/l.c.w./min
control
Ø
ØF
b
30
25
20
15
10
5
0
Rb influx uM/l.c.w./min
control
Ø
ØF

Fig 4.7 The components of basal-lateral K^+ influx measured upon subconfluent cell monolayers.

Abscissa: ^{86}Rb influx (mmole/ liter cell water/minute). K^+ influx into subconfluent cell monolayers was measured using ^{86}Rb as a tracer for K and a 5 minute influx period. Influx was measured under four experimental conditions: control; in the presence of 1mM ouabain; in the presence of 0.1mM furosemide and in the presence of 1mM ouabain plus 0.1mM furosemide. The results are expressed as the mean $\pm$ SEM. The figures in parentheses are the number of separate determinations of each condition.

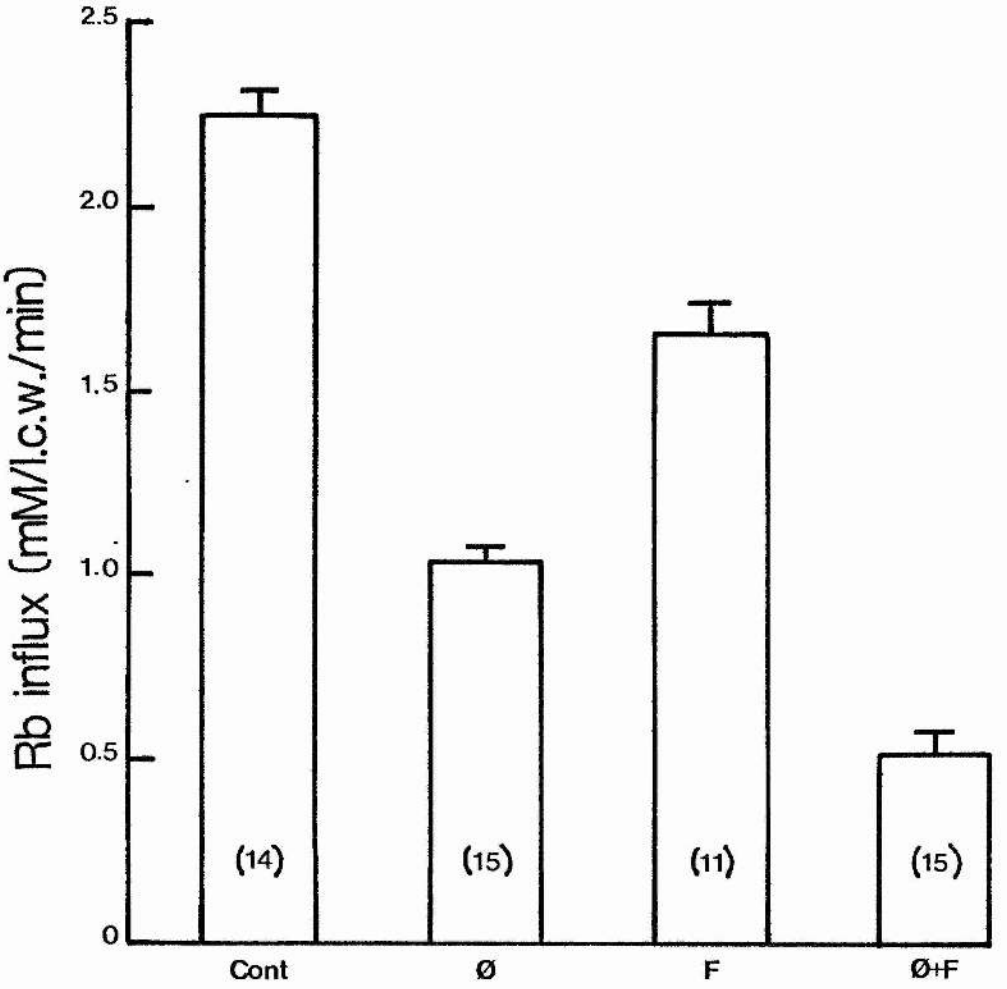

Rb influx (mM/l.c.w./min)
2.5
2.0
1.5
1.0
0.5
0
(14)
(15)
(11)
(15)
Cont
Ø
F
Ø+F

Fig 4.8 A comparison of the actions of furosemide, bumetamide and piretanide upon the ouabain insensitive component of K^+ influx.

Abscissa: ^{86}Rb influx (mmol/ liter cell water/minute). The ability of the related loop diuretics: piretanide and bumetamide to inhibit the ouabain insensitive component of K^+ influx into subconfluent cell monolayers was tested. ^{86}Rb influx was measured under control conditions, in the presence of 1mM ouabain and in the presence of 0.1mM diuretic plus 1mM ouabain. To demonstrate that the actions of loop diuretics was not additive K influx was also measured in the presence of two loop diuretics simultaneously (plus 1mM ouabain). The results are expressed as the mean $\pm$ SEM of 3 separate determinations of each condition. The results are from a single experiment.

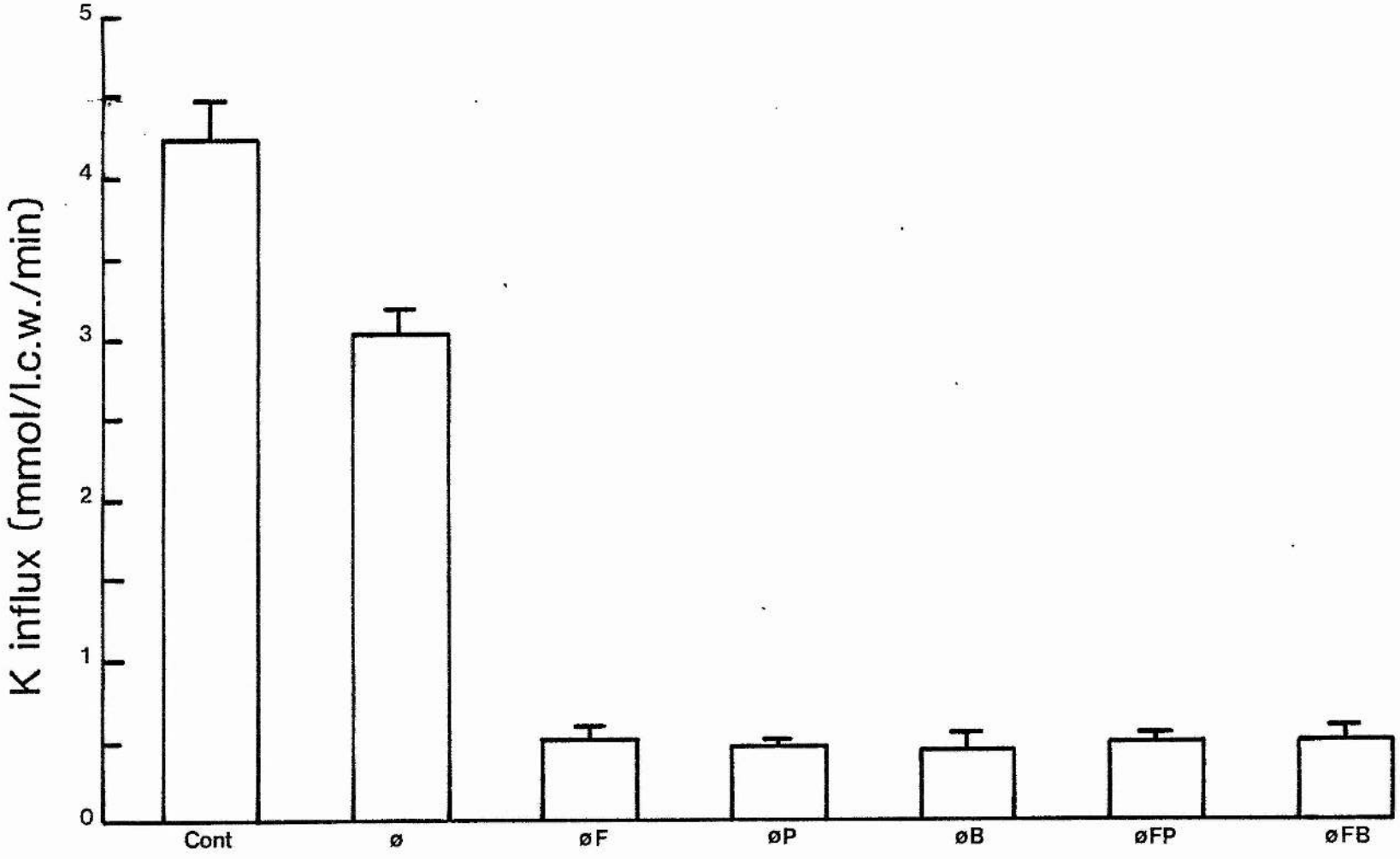
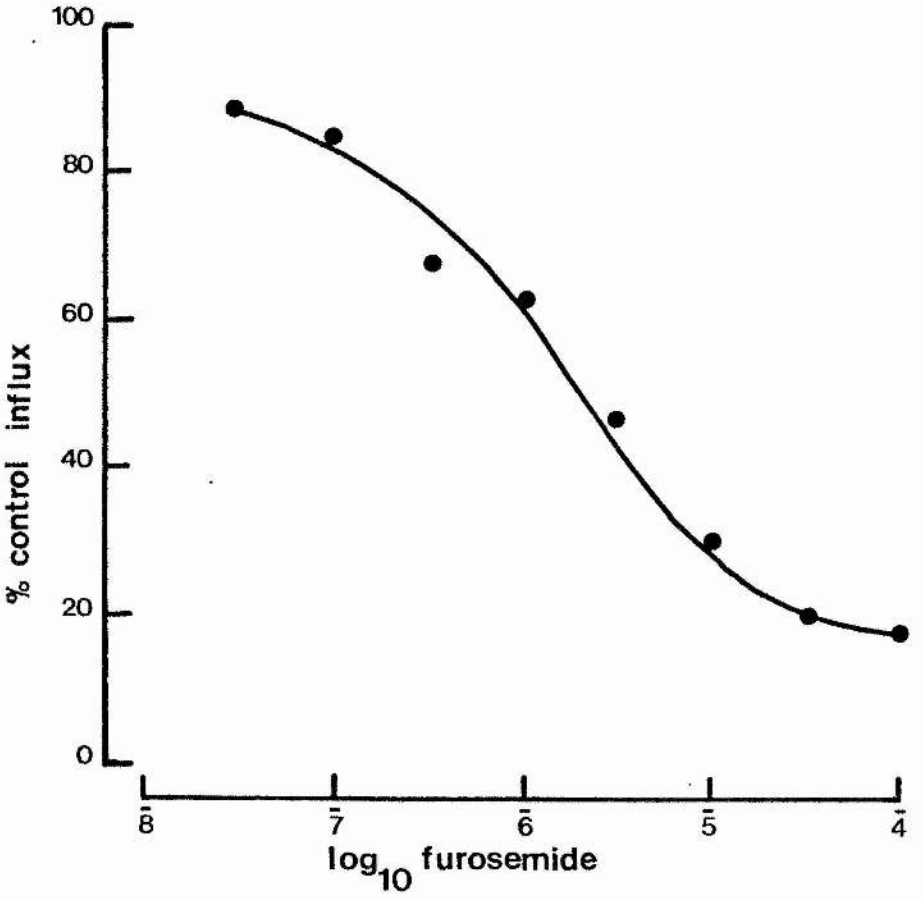

K influx (mmol/l.c.w./min)
5
4
3
2
1
0
Cont
ø
øF
øP
øB
øFP
øFB

Fig 4.9 Log dose response curve for the ability of furosemide to inhibit the ouabain insensitive component of K^+ influx.

Abscissa: percentage of control (ouabain-insensitive) ^{86}Rb influx. Ordinate: $\log_{10}$ furosemide concentration. The dose dependency of the ability of furosemide to inhibit the ouabain insensitive component of K^+ influx was tested upon subconfluent cell monolayers using ^{86}Rb as a tracer for K^+. The experiments were performed in the presence of 1mM ouabain and various concentrations of diuretic. Half maximal inhibition of the diuretic-sensitive component of influx, calculated from a Probit analysis of the dose response curve, was at $1.6 \pm 0.2 \times 10^{-6}$M furosemide. The lower graph shows a Hill plot of the log dose response curve. The solid line is the least-squares regression line and is fitted by the equation:

$$y = 0.69(x) + 4.08$$

The slope of this line is significant ($P < 0.001$) and the correlation coefficient is 0.978 ($P < 0.001$). Each datum is a single determination.


100
80
60
40
20
0
% control influx
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ furosemide

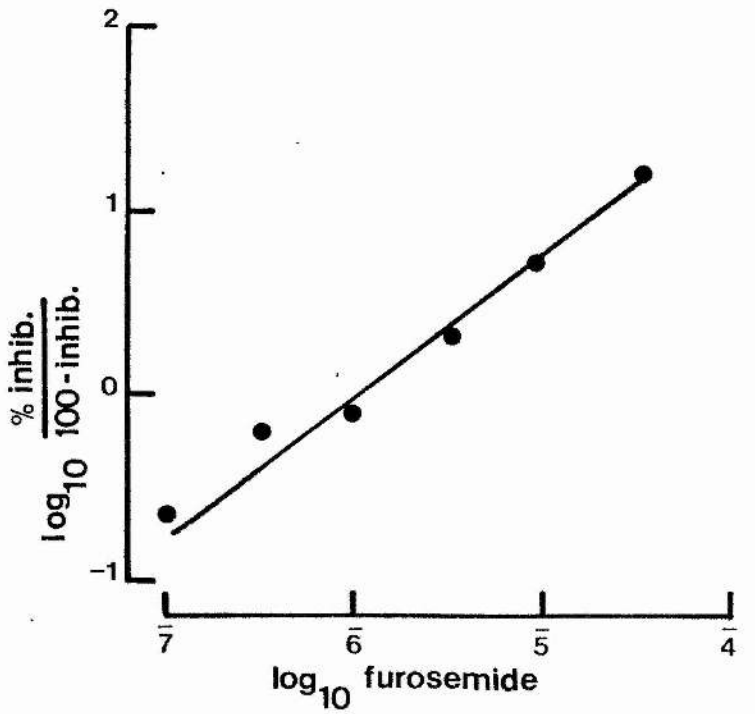
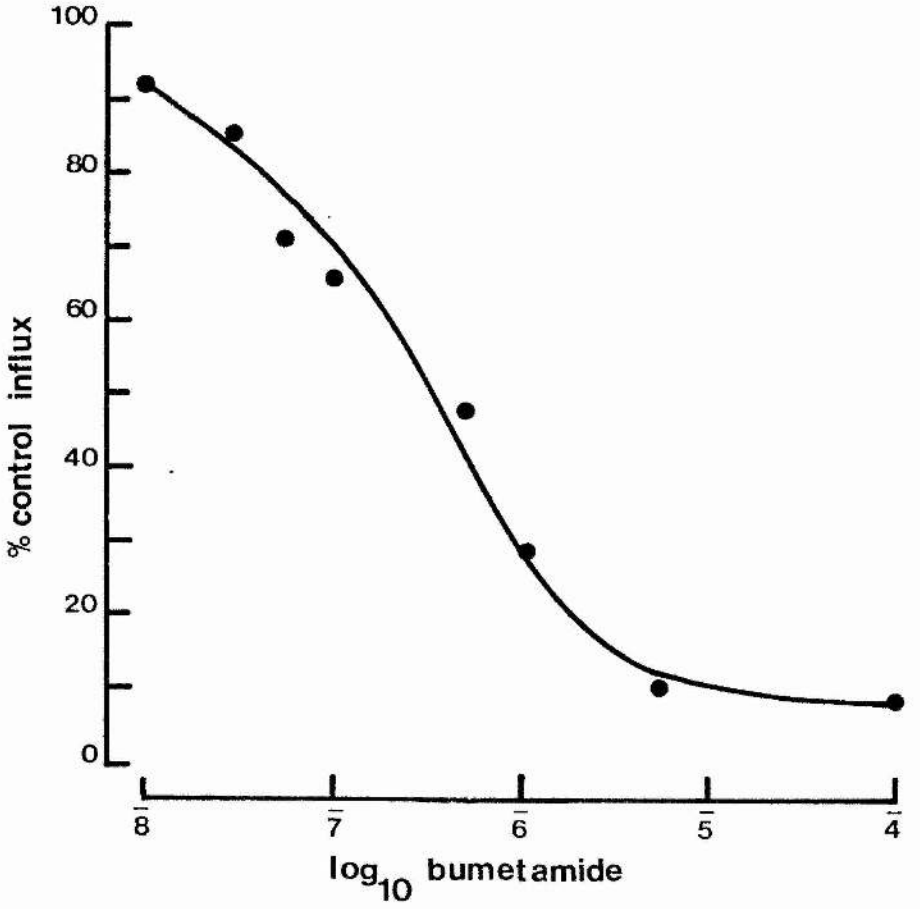

2
1
0
−1
$\log_{10} \frac{\%\ \text{inhib.}}{100 - \text{inhib.}}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ furosemide

Fig 4.10 Log dose response curve for the ability of bumetamide to inhibit the ouabain insensitive component of K^+ influx.

Abscissa: percentage of control (ouabain-insensitive) ^{86}Rb influx. Ordinate: $\log_{10}$ bumetamide concentration. The ability of bumetamide to inhibit the ouabain-insensitive component of K^+ influx was tested upon subconfluent cell monolayers grown upon plastic petri dishes. All fluxes were determined in the presence of 1mM ouabain. Half maximal inhibition of the ouabain insensitive component of influx, calculated from a Probit analysis of the log dose response curve, was found to be $0.4 \pm 0.1 \times 10^{-6}$M bumetamide. The lower graph shows a Probit analysis of the data presented in the log dose response curve. The solid line is the least-squares regression line and obeys the equation:

$$y = 0.71(x) + 4.72$$

Both the slope of this line and the correlation coefficient (0.982) are highly significant ($P < 0.001$).


100
80
60
40
20
0
% control influx
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ bumetamide

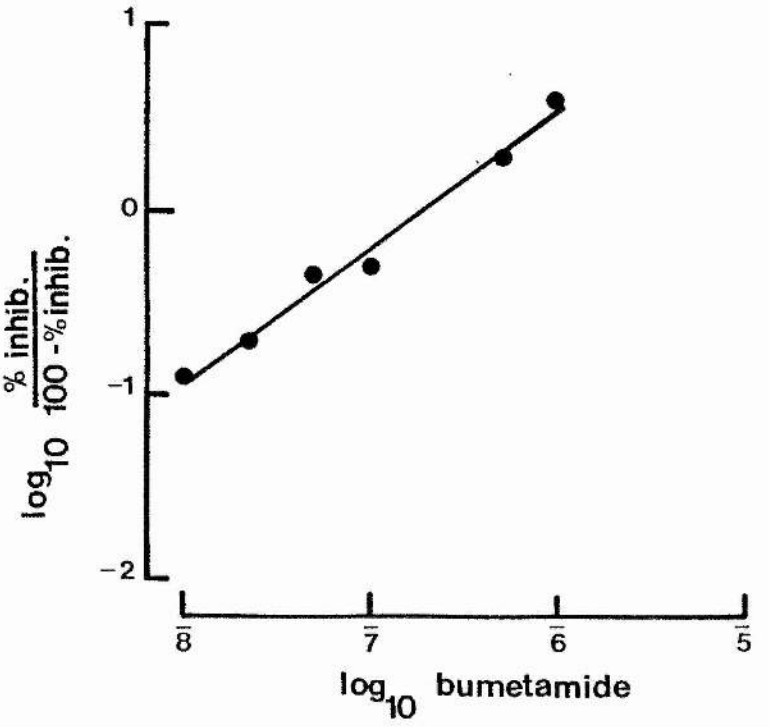
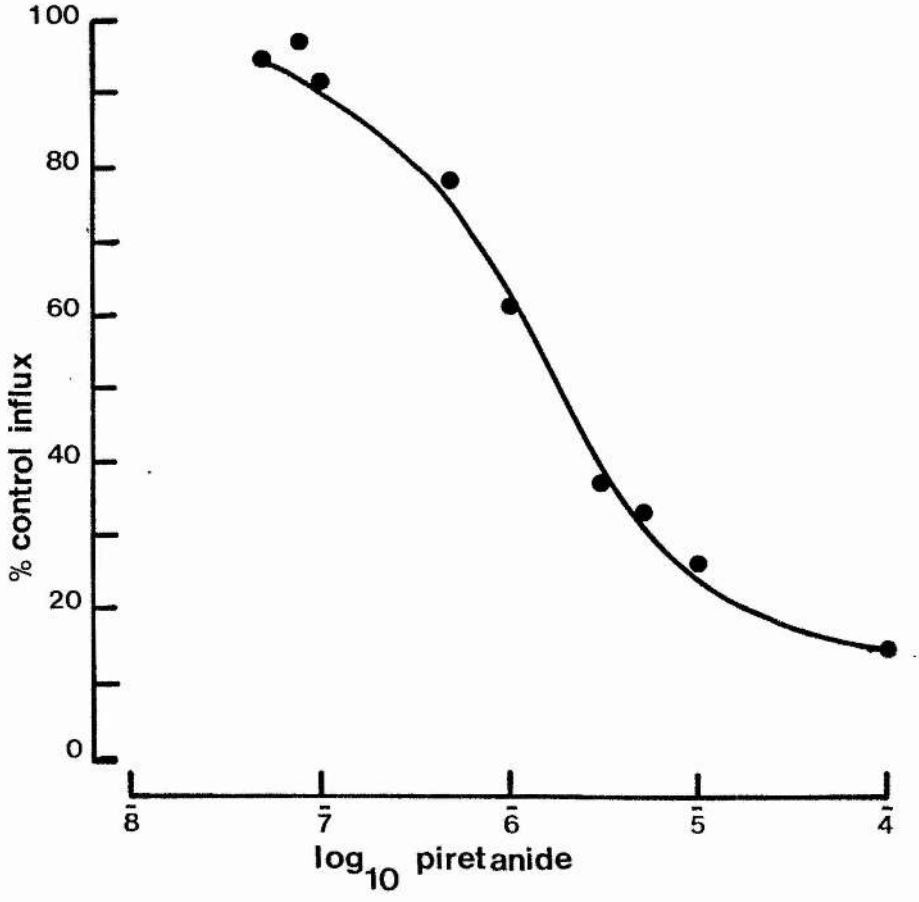

1
0
−1
−2
$\log_{10} \frac{\%\ \text{inhib.}}{100 - \%\ \text{inhib.}}$
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\log_{10}$ bumetamide

Fig 4.11 Log dose response curve for the ability of piretanide to inhibit the ouabain insensitive component of K^+ influx.

Abscissa: percentage of control (ouabain-insensitive) ^{86}Rb influx. Ordinate: $\log_{10}$ piretanide concentration. The ability of piretanide to inhibit the ouabain-insensitive component of K^+ influx was tested upon subconfluent cell monolayers. All flux measurements were made in the presence of 1mM ouabain. Half maximal inhibition of the diuretic sensitive component of K^+ influx, calculated from a Probit analysis of the data, was apparent at $1.6 \pm 0.4 \times 10^{-6}$M. The lower graph shows a Hill plot of the log dose response curve. The solid line represents the least-squares regression line and obeys the equation:

$$y = 0.89(x) + 5.34$$

Both the slope of this line and the correlation coefficient (0.995) are highly signifcant ($P < 0.001$).


100
80
60
40
20
0
% control influx
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ piretanide

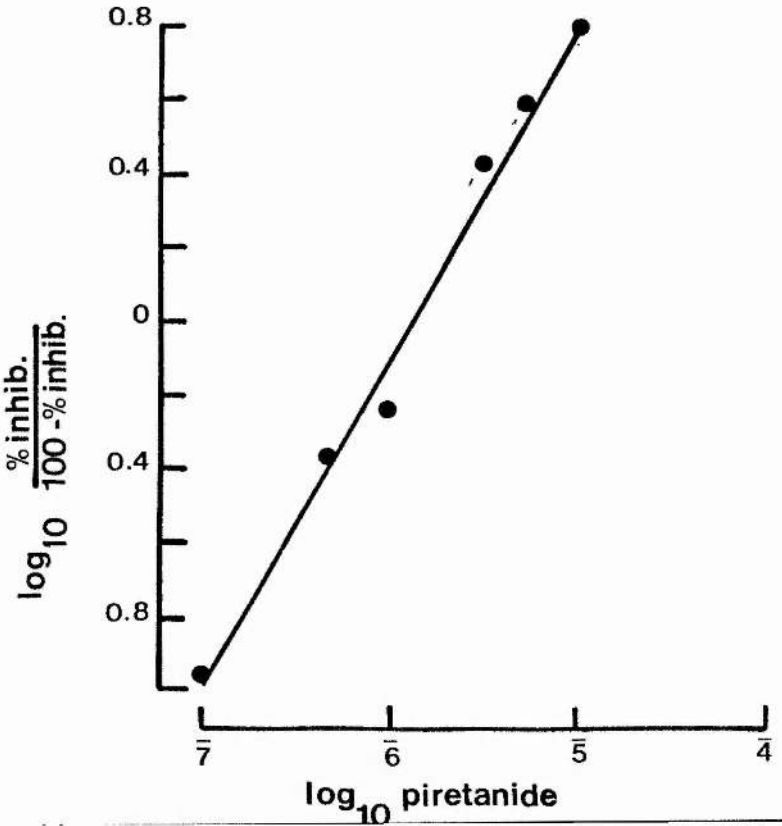

0.8
0.4
0
0.4
0.8
$\log_{10} \frac{\%\text{inhib.}}{100-\%\text{inhib.}}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ piretanide

Fig 4.12 Time-course of inhibition of K influx by furosemide.

Abscissa: percentage inhibition of total ^{86}Rb influx by 0.1mM furosemide. Ordinate: time (minutes). The time course of furosemide action was tested by measuring the furosemide sensitive component of ^{86}Rb influx into subconfluent cell monolayers after preincubation in 0.1mM furosemide for various times. To ensure an accurate measurement of ^{86}Rb influx a 5 minute flux period was used. The first time point, therefore, was 5 minutes (no preincubation). Each datum is the mean $\pm$ SEM of 3 separate determinations of influx.

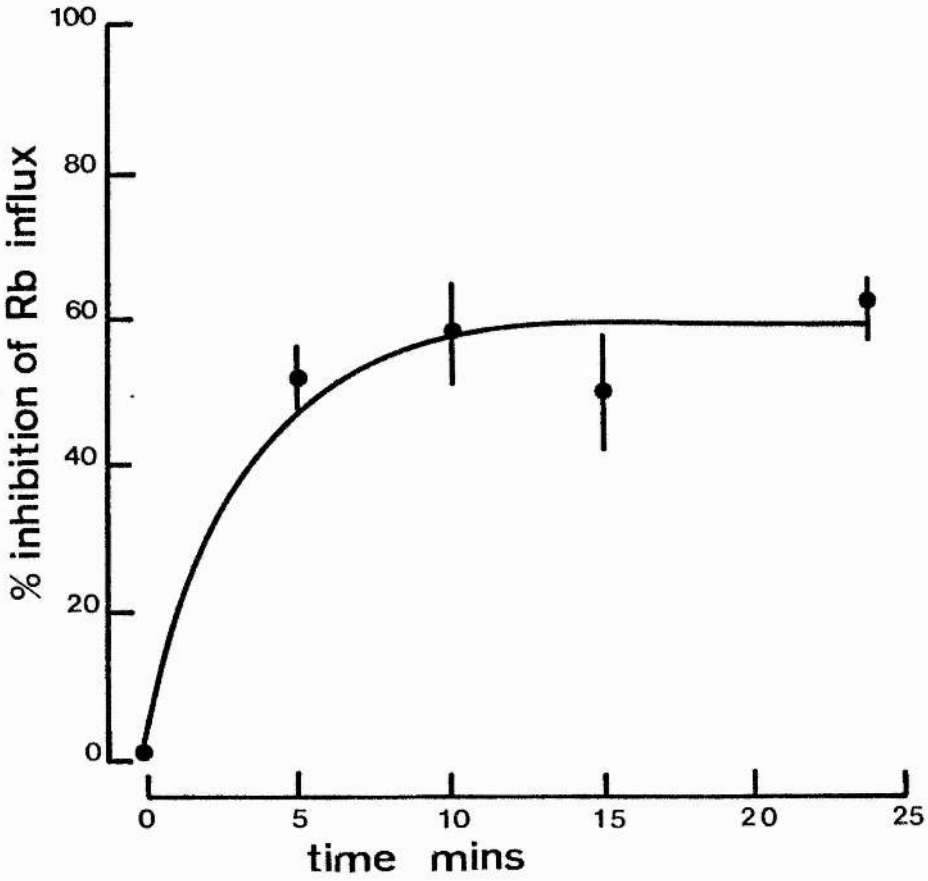

100
80
60
40
20
0
% inhibition of Rb influx
0
5
10
15
20
25
time mins

Fig 4.13 K^+ efflux across the apical and basal-lateral cell aspects of confluent MDCK cell monolayers.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: time (minutes). Epithelial cell monolayers of MDCK cells were preloaded with ^{86}Rb for 5 hours, washed x4 in Krebs and mounted into Ussing chambers. ^{86}Rb efflux across both the apical (o) and the basal-lateral cell membranes (●) was measured simultaneously, at 2 minute intervals. Efflux into the apical bathing solution has been corrected for the decrease in isotope activity in the cell during each flux period. Each datum is the mean ± SEM of 5 separate determinations.

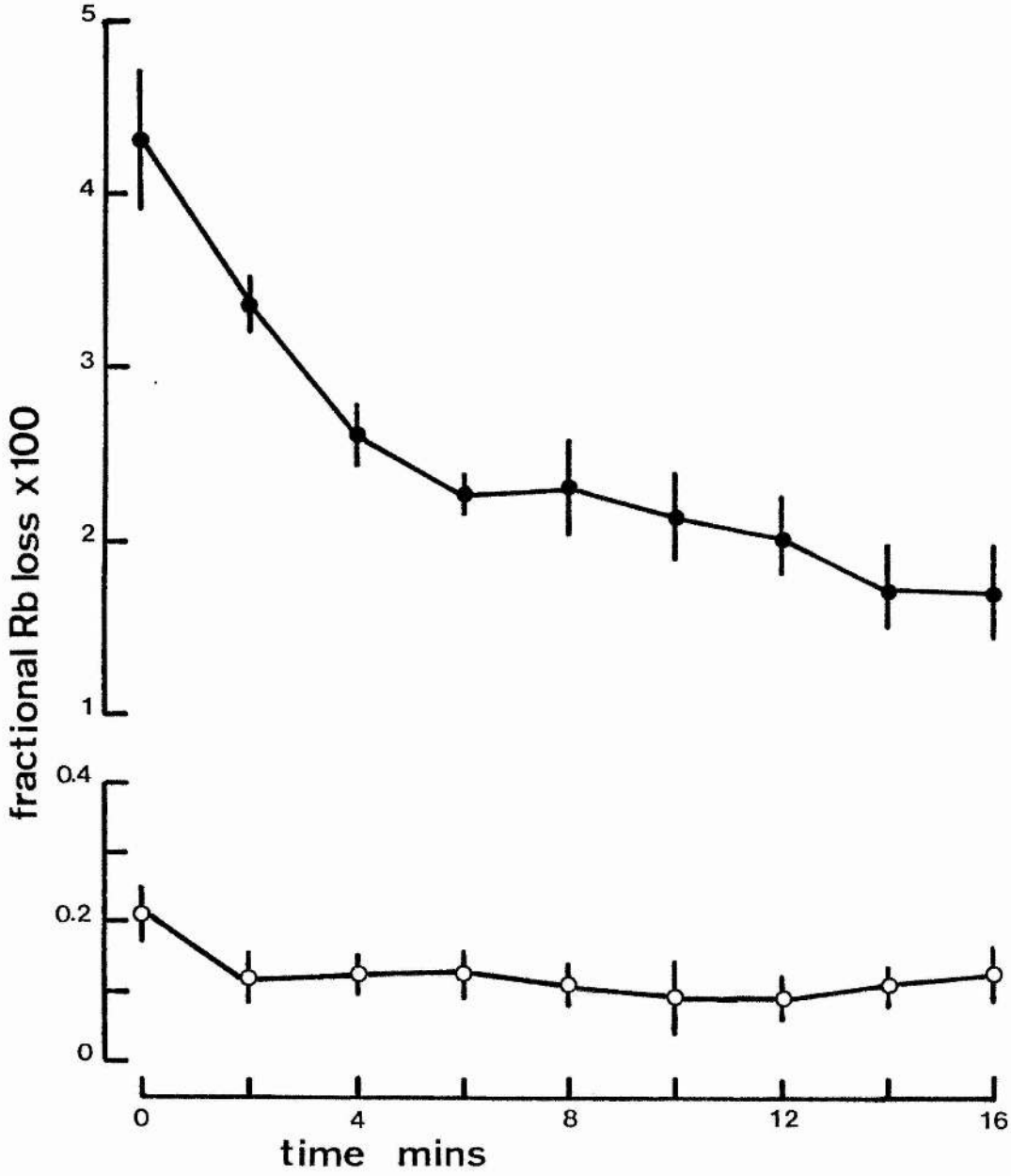
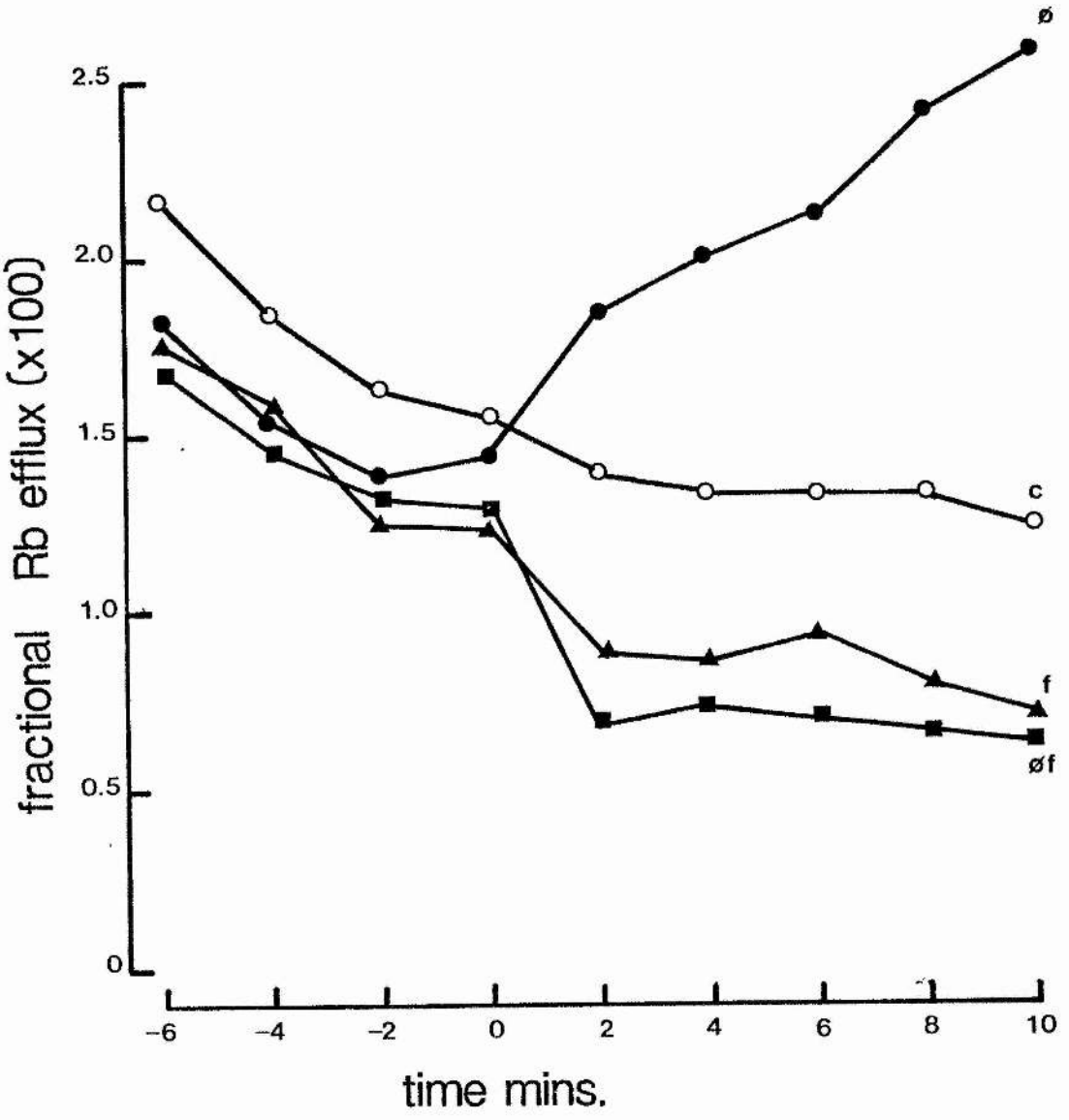

fractional Rb loss x100
5
4
3
2
1
0.4
0.2
0
0
4
8
12
16
time mins

Fig 4.14 The effects of ouabain, furosemide and ouabain plus furosemide upon K efflux from subconfluent monolayers of MDCK cells.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: time (minutes). ^{86}Rb efflux was measured in subconfluent cell monolayers of MDCK cells preloaded with ^{86}Rb for 3 hours. At t = 0 minutes either: 1mM ouabain (●), 0.1mM furosemide (▲) or 1mM ouabain plus 0.1mM furosemide (○) were added to the experimental media. A control condition was also included (■). The results are expressed as the mean of 3 separate determinations of each condition. Deviation from the mean was always less than 5%.


ø
c
f
øf
2.5
2.0
1.5
1.0
0.5
0
fractional Rb efflux (x100)
−6
−4
−2
0
2
4
6
8
10
time mins.

CHAPTER 5

EVIDENCE FOR A Ca^{2+} ACTIVATED K^{+} PERMEABILITY IN MDCK CELL MONOLAYERS

INTRODUCTION

Although it is well established that the ($Na^+ + K^+$)-ATPase is essential for both the generation of intracellular K^+ accumulation within epithelial cells (Civan, 1980) and for the establishment of active K^+ secretion in a number of mammalian epithelia (O'Neill, 1981; Schultz, 1981), comparatively little is known about either the kinetics of K^+-exchange fluxes or the properties of dissipative K^+ movements across the apical and basal-lateral cell membranes of epithelia. In an earlier chapter the kinetics of transepithelial and transcellular K^+ movements across high resistance MDCK cell monolayers have been examined and the major findings were: that the apical cell membrane was relatively impermeable to K^+; K^+-exchange fluxes being dominated by flux across the basal-lateral membrane, and that flux across the basal-lateral membranes consisted of three major flux components: A ouabain-sensitive flux mediated by the ($Na^+ + K^+$)-ATPase and responsible for intracellular K^+ accumulation (Lamb et al, 1981; Simmons, 1981d), a loop diuretic-sensitive $K^+ : K^+$-exchange component with properties (Aiton et al, 1981; Aiton et al, 1982; McRoberts et al, 1982) similar to a diuretic-sensitive $Na^+ + K^+ + Cl^-$ cotransport system described in red cells (Chipperfield, 1980) and in Ascites tumour cells (Geck et al, 1980). The third component of K^+ flux across the basal-lateral membranes was a ouabain and diuretic insensitive flux - the "ground" permeability of the membranes to K^+. In this present chapter I report that in addition to these flux components, there is a Ca^{2+}-activated K^+ permeability which when stimulated by secretagogues such as adrenaline, ATP and A23187 results in a net efflux of cellular K^+ across both

The apical and basal-lateral cell membranes.

Ca^{2+}-dependent changes in membrane permeability to K^+ were first described for red blood cells. Early studies demonstrated that metabolically poisoned red cells selectively lost large amounts of K^+ (Wilbrandt, 1937; Davson, 1941), a result ascribed to the production of abnormal metabolites. In 1958 Gardos described that this K^+ loss could be prevented by the removal of extracellular Ca^{2+} by the addition of a chelating agent (Gardos, 1958). Later studies provided convincing evidence that the gating of the K^+ permeability was controlled by the level of free intracellular Ca^{2+} (Whittam, 1968; Lew, 1970; Simons, 1976) and that the sensitivity of the gating process to intracellular Ca^{2+} was controlled by metabolic factors (for reviews see: Ferreira & Lew, 1977; Lew & Ferreira, 1978; Putney, 1979).

In recent years Ca^{2+}-dependent changes in membrane permeabilities to K^+, and in some cases to Na^+, have been reported in a wide range of vertebrate and invertebrate excitable tissue (Meech & Strumwasser, 1970; Meech, 1972; Krnjevic & Lisiewiecs, 1972; Minota, 1974; Meech and Standen, 1975; Iwatsaki & Ono, 1979; Adams et al, 1982). The physiological function of Ca^{2+}-dependent K^+ permeability in nervous tissue has recently been reviewed (Meech, 1978). Ca^{2+}-dependent changes in K^+ permeability have also been identified in non-excitable tissue including: epithelia and secretory glands, where it is involved in receptor mediated changes in K^+, and in some cases Na^+, permeability and secretion (Haylett & Jenkinson, 1972; Selinger et al, 1973; Peterson, 1976; Peterson & Ueda, 1976; Parod & Putney, 1978b; Iwatsuki & Peterson, 1978b; Atwater et al, 1979; Karashima, 1981; Moreto et al, 1981); striated and smooth muscle, where it is proposed to have a modulatory

role on membrane potential (Fink & Luttgau, 1976; Bulbring & Tomita, 1977; Pallotta et al, 1982) and in Erlich Ascites tumour cells, where it has been implicated in Na^+-dependent amino-acid transport (Valdeolmillos et al, 1982a, 1982b).

In this present chapter we present evidence for a Ca^{2+}-dependent K^+ permeability in both membranes of MDCK cells, and compare its properties with Ca^{2+}-dependent K^+ permeabilities in red blood cells and other tissue.

RESULTS

(A) Characterisation of ^{86}Rb Efflux Across the Apical and Basal-lateral Cell Boundaries: Effect of Secretagogues.

(i) Steady-state ^{86}Rb Exchange Fluxes Across the Apical and Basal-lateral Membrane Faces.

K^+ movements across confluent epithelial monolayers of MDCK cells are dominated by exchange fluxes across the basal-lateral cell membranes, flux across the apical cell aspects being of little quantitative importance. For ^{86}Rb efflux this point is illustrated in fig 5.1. ^{86}Rb efflux across the basal-lateral membranes far exceeds that across the apical membrane face: the mean fractional ^{86}Rb efflux across the apical membrane at t = 10 minutes is 0.14 ± 0.01 ($\times 10^{-2}$), n =39, compared to a mean fractional efflux across the basal-lateral membranes of 3.57 ± 0.20 ($\times 10^{-2}$), n = 39, giving a calculated mean ratio for basal-lateral : apical efflux rates of 32.9 ± 2.1. Because the system is in equilibrium, with no apparent net K^+ flux (see Chapters 3 and 4), this ratio should be the same as the ratio of basal-lateral : apical influx (~ 100). Assuming an intracellular K^+ concentration of 185 ± 2 $mmol.l^{-1}$ cell water. min^{-1} (n = 25), measured directly by flame photometry in separate control experiments, the discrepancy can be accounted for by differences in the magnitude of the apical influx and efflux. The rate of efflux calculated from isotope loss was 0.25 ± 0.02 $mmol.l^{-1}$ cell water. min^{-1} which is roughly an order of magnitude larger than the corresponding ^{86}Rb influx of 0.021 ± 0.01 $mmol.l^{-1}$ cell water. min^{-1}, because of the small magnitude of both fluxes. However these fluxes are subject to error and should be interpreted with caution.

The magnitude of ^{86}Rb efflux across the basal-lateral membrane 3.5 ± 0.4 mmol.l^{-1} cell water. min^{-1}, n = 39, is in close agreement with both the rate of ^{86}Rb influx across the basal-lateral membrane face measured previously upon identical epithelial monolayers (2.58 ± 0.21 mmol.l^{-1} cell water. min^{-1}. Chapter 4) and with the magnitude of the total ^{86}Rb influx rate into subconfluent monolayers of MDCK cells grown upon plastic petri dishes; 3.62 ± 0.34 mmol.l^{-1} cell water. min^{-1}, n = 12 (Table 6). Taken together these results confirm our earlier observations that K^+ movements across MDCK cells are dominated by flux across the basal-lateral cell aspects, similar to natural epithelia such as rabbit colon (Moreto et al, 1981).

It is also clear from fig 5.1 that the rate of fractional loss of isotope across either cell membrane declines with time before reaching a steady-state value. A semi-log_e plot of fractional ^{86}Rb loss (c.p.m.) against time (fig 5.2) shows that the rate of ^{86}Rb appearance in the basal-lateral bathing solution can be accounted for by the sum of two exponentials. The first exponential has a $t_{\frac{1}{2}}$ of 3 minutes whilst the second exponential has a $t_{\frac{1}{2}}$ of 14 minutes. The bi-exponential nature of ^{86}Rb efflux may represent loss of isotope from two compartments (Simmons, 1981d). Efflux across the apical membrane is also composed initially of loss of isotope from a cellular and extracellular compartment. With this in mind all flux measurements have been made in the period after the system reaches equilibrium.

(ii) Effects of Adrenaline, Exogenous ATP and Ionophore A23187 upon Fractional ^{86}Rb Efflux Across the Apical and Basal-lateral Cell Aspects.

Addition of 100 uM adrenaline (fig 5.1) stimulates an increase in ^{86}Rb efflux across both membrance faces although, like the steady-state

isotope exchange, the increase in flux across the basal-lateral membrane face is of greatest quantitative importance, the increase in isotope loss across the apical membrane is still significant ($P < 0.01$, Table 5.1). The adrenaline stimulated increase in ^{86}Rb efflux across both membranes was transient; the peak increase in ^{86}Rb efflux rate was observed four minutes after adrenaline addition (Table 5.1) after which time a time-dependent decrease in the efflux rate, in the continued presence of adrenaline, was evident. Similar secretagogue-stimulated transient increases in cellular K^+ effluxes have been reported in a number of tissues including epithelia (for reviews see: Jenkinson et al 1978; Putney, 1979). In contrast to the ability of adrenaline applied to the basal-lateral bathing solution to stimulate ^{86}Rb efflux, addition of an equi-molar concentration (100 uM) to the apical bathing solution had no effect upon either the apical or basal-lateral efflux rates (fig 5.1) - mean data is shown in Table 5.1. These results suggest that the adrenoreceptors mediating the adrenaline stimulated ^{86}Rb efflux are located solely upon the basal-lateral membranes - a result in agreement with the localisation of alpha- and beta-receptors to the basal-lateral membrane for adrenaline stimulated rheogenic Cl^- secretion (Chapter 3). Fig 5.3 shows that addition of 100 uM ATP to the basal bathing solution stimulates a similar transient increase in fractional ^{86}Rb efflux as adrenaline although its efficacy appears to be greater. In contrast to the action of adrenaline, exogenous ATP is equally effective if applied to either bathing solution (fig 5.3, Table 5.1). Exogenous ATP has been reported to stimulate a rheogenic Cl^- secretion in this cell-line and the response is thought to be mediated through purinergic (P_2) receptors located on both membrane faces (Simmons, 1981b, 1981c). A P_2

receptor mediating an ATP induced Ca^{2+}-dependent K^+ efflux has recently been reported in parotid cells (Gallacher, 1982). In several epithelia a raised intracellular Ca^{2+} level has been implicated as an activator of passive K^+ efflux (Iwatsuki & Peterson, 1978a, 1978b; Putney, 1979; Moreto et al, 1981). A similar role for Ca^{2+} ions in the activation of ^{86}Rb efflux across both the apical and basal-lateral membranes of MDCK cells is suggested from the data presented in fig 5.4 which shows that the Ca^{2+} ionophore A23187 (Reed & Lardy, 1972; Pressman, 1973, 1976) stimulates ^{86}Rb efflux across both cell membranes when added to the apical bathing solution. Addition to the basal-lateral bathing solution (fig 5.4, Table 5.1) was without effect upon the steady-state ^{86}Rb efflux rate across either cell membrane ($P > 0.5$ for each flux, Table 5.1).

(B) Characterisation of the Adrenaline, ATP and A23187 Stimulated ^{86}Rb Efflux Across the Basal-lateral Cell Aspects.

(i) Comparison of Epithelial and Subconfluent Preparations.

Since ^{86}Rb efflux across the basal-lateral membrane face under both control and drug-stimulated conditions is of greatest quantitative importance (see above), measurements of ^{86}Rb efflux from subconfluent monolayers of MDCK cells grown upon plastic petri dishes will consist primarily of efflux across the basal-lateral membrane and provide a cheap and more convenient experimental preparation with which to characterise the pharmacology and cellular mechanism of secretagogue induced K^+ efflux across the basal-lateral membrane. Fig 5.5 shows that ^{86}Rb efflux from subconfluent cell monolayers of MDCK cells can be fitted by a single exponential. The $t_{\frac{1}{2}}$ of 14.4 mins is similar to the

second exponential efflux rate constant from confluent cell monolayers (14 min). Fig 5.6 shows that application of adrenaline (100 uM) stimulates a transient increase in ^{86}Rb efflux which was quantitatively similar to that observed from confluent monolayers. The mean peak increase in ^{86}Rb efflux from plates was 2.32 ± 0.3, $n = 30$ (t_2/t_0) compared to a ratio of 2.28 ± 0.3 (t_4/t_0), $n = 6$, from confluent cell monolayers. Similar results were also obtained for ATP-stimulated (fig 5.7) and A23187-stimulated (fig 5.8) ^{86}Rb effluxes, suggesting that subconfluent monolayers are a suitable preparation with which to study some aspects of ^{86}Rb efflux across the basal-lateral membrane.

(ii) Pharmacological Characterisation of Adrenoreceptors Sub-type.

In a previous chapter (Chapter 3) both alpha- and beta-adrenoreceptors were implicated in the modulation of transepithelial Cl^- transport. Fig 5.9 shows that adrenaline stimulation of ^{86}Rb efflux is a saturable process; half maximal stimulation of efflux is achieved with $9.1 \pm 0.2 \times 10^{-7}$M adrenaline which is significantly different from the concentration of adrenaline which stimulates Cl^- secretion to half its maximal value ($3.1 \pm 0.3 \times 10^{-8}$M). Table 5.2 shows the effect of various adrenoreceptor agonists and antagonists upon ^{86}Rb efflux. ^{86}Rb efflux was stimulated by the alpha-receptor agonist phenylephrine, the maximum response being obtained with 1×10^{-4}M phenylephrine. Isoprenaline, a specific beta-adrenoreceptor agonist, had no effect upon ^{86}Rb efflux rates, as did the relatively specific $alpha_2$-agonist oxymetozaline (Lees, 1981). Table 5.2 also shows that adrenaline-stimulation of ^{86}Rb was abolished in the presence of the alpha-receptor antagonist phentolamine, yet was potentiated ($P < 0.01$ against adrenaline alone) in the presence of the beta-receptor antagonist propranolol. The mechanism by

which propranolol potentiates adrenaline-stimulated ^{86}Rb efflux is unclear. It may be the result of beta-adrenoreceptor antagonism of the alpha-receptor stimulated ^{86}Rb efflux (but see below). A direct stimulatory effect of propranolol upon steady-state ^{86}Rb efflux, as reported for human red blood cells (Lew & Ferreira, 1978), is considered unlikely since propranolol had no stimulatory effects upon ^{86}Rb efflux in the absence of adrenaline (mean results: control efflux ratio (t_2/t_0) was 0.98 $\pm$ 0.03, n = 4 and plus 1 uM propranolol 1.00 $\pm$ 0.12, n = 4, P $>$ 0.5). Taken together these results imply that catecholamine-stimulation of ^{86}Rb efflux is mediated by alpha-adrenoreceptors of the $alpha_1$ sub-type (Berthelson & Pettinger, 1977; Lees, 1981), located upon the basal-lateral cell membranes of MDCK cells. Similar activation of membrane K^+-permeability by alpha-adrenoreceptor stimulation have been reported in a number of other tissues including epithelia (Haylett, 1976; Strittmatter et al, 1977; Bulbring & Tomita, 1977; Parod & Putney, 1978).

Fig 5.10 shows a log dose response curve for the ability of exogenous ATP to stimulate ^{86}Rb efflux; half maximal stimulation of efflux by ATP occurs at 4.4 $\pm$ 0.4 x 10^{-6}M, n = 11, a value similar to that for adrenaline, and to that reported for the half maximal stimulation of K^+ efflux by ATP in cultured HeLa cells (Aiton & Lamb, 1980). A purine receptor has previously been identified in MDCK cells, stimulation of which with ATP generates a net Cl^- secretion with an apparent affinity constant of 1.9 x 10^{-5}M (Simmons, 1981b, 1981c). Fig 5.11 shows that the ionophore A23187 also stimulates ^{86}Rb efflux in a dose dependent manner.

(iii) Pharmacology of the Secretagogue-induced K^+ Permeability Across the Basal-lateral Membrane

In the majority of the systems so far investigated activation of membrane K^+ conductance by secretagogues (in the case of epithelia) or neurotransmitters is generally dependent upon the presence of Ca^{2+} ions in the extracellular media. Table 5.3 shows that a large proportion (75%) of the adrenaline-stimulated ^{86}Rb efflux was abolished in a nominally Ca^{2+}-free media, prepared by the omission of $CaCl_2$ and the addition of 2mM EGTA. A similar Ca^{2+} dependence (60%) of the efflux response was observed with ATP (Table 5.4). In both cases a significant ($P < 0.01$ and $P < 0.001$, for adrenaline and ATP stimulation respectively) increase in ^{86}Rb efflux was however still observed. These results suggest that for maximal stimulation of ^{86}Rb efflux by adrenaline or ATP requires an influx of extracellular Ca^{2+} resulting in an increase in intracellular free Ca^{2+} levels as has been reported in other cells (Haylett, 1976; Iwatsuki & Peterson,1978). The small stimulation of efflux remaining in a Ca^{2+}-free media (Tables 5.3, 5.4) may be explained either as a result of receptor-mediated release of Ca^{2+} from intracellular stores (Weiss & Putney, 1978; Karashima, 1981) or of the inability to completely remove Ca^{2+} from the bathing media in intimate contact with the cell layer. The hypothesis that the rise in membrane K^+ conductance is the result of a receptor-mediated rise in intracellular free Ca^{2+} is supported by the finding that the Ca^{2+} ionophore is an effective activator of ^{86}Rb efflux (fig 5.4). Table 5.4 shows that the actions of A23187 were dependent upon the presence of extracellular Ca^{2+} and not the result of a non-specific membrane effect (Selinger et al, 1974; Reed, 1976; Parod & Putney, 1978b).

Ca^{2+}-activated K^+ permeability in red blood cells and a number of other tissues is sensitive to inhibition by quinine at relatively high concentrations (0.1 - 5mM) (Armando-Hardy et al, 1975; Atwater et al, 1979; Burgess et al, 1981). Table 5.3 shows that 1 mM quinine is an effective inhibitor of the adrenaline activated ^{86}Rb efflux. Similar results were obtained for ATP (Table 5.4). That the inhibitory action of quinine was attributable, at least in part, to the inhibition of the K^+ permeability mechanism rather than antagonism of receptor occupation by adrenaline or ATP (Mecca et al, 1980; Simmons, 1981b) was supported by the observations that (1) an equi-molar concentration of quinine also blocked the A23187 stimulated ^{86}Rb efflux (Table 5.4 and see Burgess et al, 1981) and (2) addition of quinine to the apical bathing solution of confluent epithelial monolayers inhibited the response to basal-lateral application of adrenaline (Table 5.8). Apamin, a polypeptide isolated from bee venom (Haberman, 1972), a potent (in the nanomolar range) inhibitor of Ca^{2+}-activated K^+ movements in isolated hepatocytes (Burgess et al, 1981) had no significant inhibitory actions upon the adrenaline (Table 5.3) or the ATP (Table 5.4) stimulated ^{86}Rb efflux when used either at 25nM or 250nM. Apamin is also ineffective in inhibiting Ca^{2+}-activated K^+ transport in red blood cells (Burgess et al, 1981), suggesting that there may be several pharmacological distinct Ca^{2+}-activated K^+ channels in different tissues. Tetraethylammonium (25mM), whilst having no significant inhibitor actions upon the magnitude of the resting ^{86}Rb efflux rate was an effective blocker of both the adrenaline (Table 5.3) and the ATP stimulated ^{86}Rb efflux (Table 5.4) as found in red blood cells (Simons, 1976), vertebrate sympathetic ganglia (Adams et al, 1982) and K^+ efflux across the mucosal

border of rabbit colon (Moreto et al, 1981).

Incubation of the cells with 1 mM ouabain had opposing effects upon the size of the adrenaline and ATP stimulated ^{86}Rb efflux; ouabain caused a significant inhibition of the adrenaline stimulated efflux (Table 5.4) but a significant stimulation of the ATP-activated ^{86}Rb efflux. In human red cells ouabain blocks the Ca^{2+}-activated K^+ permeability (Lew, 1971). This action is not thought to arise directly from inhibition of the $(Na^+ + K^+)$-ATPase but from secondary effects (Karlish et al, 1981), possibly by changes in intracellular ATP/ADP levels affecting passive membrane ion permeabilities (Cotterrell & Shields, 1982) or active Ca^{2+} extrusion. The opposing effects of ouabain upon the adrenaline and ATP stimulated K^+ permeability might be explained in terms of an effect of ATP upon the intracellular ATP/ADP ratios. The loop diuretic furosemide, as previously reported (Chapter 4), was able to reduce the control ^{86}Rb efflux (Table 5.3) by inhibition of passive Na + K + Cl co-transport, but had no effect upon either the adrenaline or ATP stimulated ^{86}Rb effluxes (Table 5.3, 5.4) in contrast to its inhibitory actions in red cells (Blum & Hoffman, 1971). In Table 5.2 we described that propranolol potentiated adrenaline stimulation of ^{86}Rb efflux and that the action may have been mediated by beta-blockade (decrease in intracellular cAMP levels). Table 5.3 shows that incubation with 1 mM isomethylbutylxanthine (IBMX), a procedure which raises intracellular cAMP levels (Rugg & Simmons, 1982), had no effect upon either control, adrenaline stimulated, or ATP stimulated (Table 5.4) ^{86}Rb efflux.

(iv) Inactivation of the Secretagogue-induced ^{86}Rb Permeability

Even in the continued presence of the secretagogue, stimulation of ^{86}Rb efflux across both cell membranes is transient (figs 5.1, 5.3, 5.4).

Fig 5.12 shows that a brief exposure to adrenaline caused a similar transient response as a continued application (fig 5.1) although upon washing free the adrenaline, the ^{86}Rb efflux rate decreased towards pre-stimulation levels quicker than in the presence of adrenaline. A second exposure to an equi-molar concentration (100 uM) after six minutes was then ineffective in stimulating ^{86}Rb efflux. In contrast, addition of 20 uM A23187 was equally effective in stimulating fractional ^{86}Rb loss from tissues pre-treated with adrenaline as it was from cells exposed only to A23187 (fig 5.12). The results from this experiment can be interpreted in two ways: (1) The desensitisation of the response to a second challenge with adrenaline results from a receptor-mediated decrease in sensitivity to adrenaline, as has been proposed for rat parotid cells where desensitisation of K^+ efflux to adrenaline is coupled to a decrease in alpha-receptor numbers (Strittmatter at al, 1977b). (2) Desensitisation results from a Ca^{2+}-dependent inactivation of Ca^{2+} channels as proposed to occur in a number of tissues including parotid glands (Putney, 1979; Standen, 1981), but not from a time-dependent inactivation of K^+-channels (Brown & Lew, 1981; Garcia-Sancho et al, 1982). Although the A23187 result favours the first interpretation, A23187 has been reported to have a direct effect upon the Ca^{2+} sensitivity of K^+ channels in sickle cell anaemia where it activates K^+ efflux from red cells with high internal free Ca^{2+} and K^+ channels refractile to activation by Ca^{2+} (Lew & Bookchin, 1980). Support for the second interpretation, Ca^{2+}-dependent inactivation of K^+ channels is shown in fig 5.13, which shows that adrenaline is ineffective in stimulating fractional ^{86}Rb efflux after pre-exposure to ATP (100 uM). The decrease in fractional ^{86}Rb efflux in the continued

presence of ionophore A23187 may result from either intracellular Ca^{2+} buffering, or to export of Ca^{2+} from the cell (Lew & Ferreira, 1978).

(v) Stimulation of a Net K^+ Loss by Adrenaline and ATP.

Stimulation of K^+ movements in many (Strittmatter et al, 1977a; Burgess et al, 1981; Moreto et al, 1981), but not all (Aiton & Lamb, 1980; Burgess et al, 1981), tissues by secretagogues results in a net loss of cellular K^+. That adrenaline and ATP stimulate a similar net flux in MDCK cells can be demonstrated in two ways. Firstly, table 5.5 shows that in identical subconfluent monolayers 100 uM adrenaline had no stimulatory effect upon either the magnitude of the total ^{86}Rb influx or upon its distribution between the ouabain-sensitive, furosemide-sensitive and ouabain and furosemide sensitive components of influx. Similar results were obtained for the effect of ATP upon ^{86}Rb influx. A net K^+ loss from the cells can also be demonstrated directly - by measuring net K^+ cellular ion contents in the presence and absence of adrenaline or ATP by flame photometry (Table 5.6). The internal and K^+ concentrations under control conditions were of a similar order to those previously reported for MDCK cells (Aiton et al, 1982). Addition of 100 uM adrenaline resulted in a rapid loss of cellular K^+ over the first five minutes, followed by a recovery of K^+ contents towards control levels.

The time course of cellular K^+ loss in the presence of adrenaline, as measured by flame photometry, closely mirrors that measured by ^{86}Rb efflux. A similar loss of cellular K^+ in the presence of ATP has been reported by Simmons (1981d) who also observed an ATP mediated net loss of cellular Na^+ and a significant decrease in cell volume. In a number of tissues secretagogue-stimulated K^+ efflux is accompanied by a parallel

increase in Na^+ influx and a stimulation of Na^+-pump activity - responsible for the delayed hyperpolarisation (Putney & Parod, 1978; Thaysen, 1978; Gallacher, 1982). Table 5.5 shows that for MDCK cells secretagogues have no significant effect upon the activity of the Na^+-pump and, coupled with the observations of Simmons (1981d), these results imply that secretagogue induced changes in membrane permeability may be restricted to K^+. Direct measurement of small changes in cellular Na^+ concentrations by flame photometry is however problematic.

(vi) Comparison of K^+-movement Across the Basal-lateral Cell Aspects of Strain I and Strain II MDCK Cells.

Two separate strains of MDCK cells have been identified that exhibit differing morphological, biochemical and electrophysiological properties (see Chapter 1 and Richardson et al, 1981; Barker & Simmons, 1981). A comparison of the actions of adrenaline, ATP and A23187 upon low electrical resistance strain II cells and high resistance strain I cells, upon which all previous measurements in this chapter have been made, is shown in Table 5.7. The results show that strain II cells possess a similar Ca^{2+}-activated K^+ permeability which can be stimulated directly with A23187 or through membrane receptors with ATP. In contrast to strain I cells adrenaline is ineffective in stimulating fractional ^{86}Rb loss from strain II cells. Since ATP has an unchanged efficacy this may imply that strain II cells lack functional alpha-adrenoreceptors. As with strain I cells a significant proportion of the A23187 activated fractional ^{86}Rb loss is independent of extracellular Ca^{2+}.

(C) Characterisation of Secretagogue Stimulated ^{86}Rb Efflux Across The Apical Cell Aspects.

Although quantitively efflux across the basal-lateral cell border is of more importance, adrenaline, ATP and A23187 stimulate a small but significant ($P < 0.01$ for each drug) increase in fractional efflux across the apical cell border (Table 5.1). In contrast to efflux ^{86}Rb influx across the apical border is depressed (control influx = 0.021 $\pm$ 0.002 mmol. l^{-1} cell water. min^{-1} compared to 0.013 $\pm$ 0.001 mmol. l^{-1} cell water. min^{-1} plus adrenaline, $n = 4$ for each condition, $P < 0.01$). There are two important points to establish about the efflux across the apical border: Firstly, does it represent a true Ca^{2+}-activated permeability in the apical cell membrane or is it merely the result of K^+ leakage from the basal bathing solution through the apical tight junction? And secondly, do the pharmacological properties of the two conductances differ?

Evidence in favour of K^+ movement across the apical cell aspects proceeding by a transcellular route includes (i) the small size of the transepithelial K^+ permeability P_{b-a} was $8.88 \times 10^{-3} cm.hr^{-1}$ (see Chapter 4), and (ii) that modulation of junctional integrity, and hence the magnitude of passive paracellular ion movements, has no effect upon the rate of fractional ^{86}Rb loss across the apical cell aspects. This point is illustrated in fig 5.14 which shows that removal of Ca^{2+} from the bathing media, by omission of $CaCl_2$ from the Krebs' buffer, results in a rapid fall in transepithelial electrical resistance to about 40% of its initial value within the first fifteen minutes in a Ca^{2+}-free media, attributed to disassembly of apical tight junctional complexes and concomitant increases in the paracellular conductance pathway

(Cereijido et al, 1978; Martinez-Palomo et al, 1980; Cereijido et al, 1981). If a large proportion of the K^+ efflux was via the paracellular route then one might expect that large changes in the conductance of this pathway might be mirrored by changes in the amount of isotope appearing in the apical bathing solution. Fig 5.14 shows that this is clearly not the case since there is no significant change in the rate of isotope exchange across the apical cell border despite an almost 40% change in resistance.

To compare the pharmacological characteristics of both the apical and basal-lateral ^{86}Rb effluxes the effects of various experimental procedures upon efflux across either cell border were investigated. Table 5.8 shows that although the adrenaline stimulated efflux across the apical membrane was sensitive to inhibition by both apically applied quinine and TEA, these drugs simultaneously inhibited efflux across the basal-lateral cell membrane. Similarly, drugs applied to the basal-lateral cell membrane also affected flux across the apical cell membrane. Thus it was not possible to distinguish different pharmacological characteristics between the two efflux pathways. Since the cell monolayers have a low paracellular shunt pathway these results imply that quinine and TEA have a significant transcellular permeability.

Table 5.8 also shows that a major part of the adrenaline activated apical ^{86}Rb efflux is sensitive to the transepithelial voltage since clamping the membrane potential to 0 mV results in a marked decrease in the adrenaline stimulated ^{86}Rb efflux. The open circuit potential four minutes after adrenaline application was 11.04 ± 0.98 mV, $n = 4$. The lack of effect of voltage clamping upon the adrenaline stimulated basal-lateral efflux is consistent with the relatively high apical

membrane resistance (Simmons, 1981a). Current passage across the epithelium will therefore predominately affect the apical membrane potential (Fromter & Gebler, 1977).

DISCUSSION

A K^+-permeability activated by raised intracellular Ca^{2+} levels has been previously described in a number of tissues including epithelia (see introduction for references). The evidence presented in this chapter suggests that a Ca^{2+}-dependent K^+ permeability is present in both the apical and basal-lateral plasma membranes of MDCK cells. The conductance at both membranes can be activated physiologically by adrenaline acting on an alpha-adrenoreceptor, or by exogenous ATP, or by raising intracellular Ca^{2+} levels artificially with the divalent cation ionophore; A23187.

The pharmacological characteristics of the secretagogue-stimulated K^+ efflux are similar to those reported in other tissues, and notably red blood cells: The K^+-conductance is activated by processes that raise intracellular Ca^{2+} levels. Cyclic nucleotides appear to play no role in activating the channel since both isoprenaline and IBMX, effective stimulators of intracellular cAMP levels in MDCK cells (Rugg & Simmons, 1982), are ineffective in activating K^+ efflux. The strongest evidence for a role for Ca^{2+} is that removal of Ca^{2+} from the extracellular media results in a very diminished response to any of the secretagogues (Lew & Ferreira, 1978; Putney, 1979). The conductance was inhibited by both quinine and tetraethylammonium Cl, but was insensitive to inhibition by apamin and furosemide, although furosemide significantly reduced the steady-state K^+ efflux. Ouabain significantly reduced the adrenaline stimulated K^+ efflux, as seen in red blood cells (Lew, 1971; Karlish *et al*, 1981), yet significantly increased the rate of efflux in the presence of ATP. These divergent effects of ouabain are difficult

to reconcile with the idea of a single mechanism of increasing K^+ conductance after receptor occupation and may imply that exogenous ATP may enter the cell and in some way alter metabolism although the mechanism is not clear.

A comparison of the effects of adrenaline, ATP and A23187 upon strain I and strain II MDCK cells shows further differences between the two cell types. In strain I cells, on which most of the experiments in this thesis were performed, adrenaline, ATP and A23187 stimulated K^+ efflux across both cell membranes, and the localisation of adrenoreceptors to the basal-lateral and ATP-receptors on both plasma membrane is in agreement with earlier measurements (Chapter 3 and Richardson et al, 1981). Why A23187 was only effective from the apical bathing solution is not clear, but may be the result of access problems to the basal-lateral cell aspects through the filter support. Although strain II cells appear to exhibit a Ca^{2+} dependent K^+ permeability, stimulated by both ATP and A23187, they do not seem to possess functional adrenoreceptors. A similar conclusion was reached previously by Richardson and co-workers on the basis that no short circuit current response to adrenaline was seen in strain II cells (Richardson et al, 1981).

Three main mechanisms have been proposed for the transient nature of drug stimulated Ca^{2+}-dependent K^+ efflux in a number of tissues; firstly in parotid glands a time and concentration dependent decrease in receptor numbers has been proposed to explain the decrease in K^+ efflux upon continued exposure to adrenaline (Strittmatter et al, 1977a, 1977b). The second, and most widely accepted mechanism, is that as intracellular Ca^2 levels rise there is a concurrent Ca^{2+}-dependent decrease in the sensitivity of the K^+ gating mechanism to Ca^{2+} (for reviews see: Lew &

Ferreira, 1978; Putney, 1979). The third proposed mechanism is that the Ca^{2+}-activated K^+ channels are voltage sensitive (Adams et al, 1982; Pallotta et al, 1981). For MDCK cells the most likely explanation for the transient nature of the efflux is that there is a Ca^{2+} dependent decrease in sensitivity of the channel to intracellular Ca^{2+}. A role for receptor-desensitisation would seem unlikely since pre-exposure to ATP also blocks the response to a second agonist (adrenaline). The transient nature of the K^+ efflux to A23187 may result from either intracellular Ca^{2+} buffering or to Ca^{2+} export from the cell (Valdeolmillos et al, 1982a). A second challenge by A23187 on a cell layer refractory to adrenaline results in an identical response which may be due to an effect of ionophore upon the sensitivity of the gating mechanism to Ca^{2+} (Lew & Bookchin, 1981).

It may be worthwhile at this point to speculate about the physiological importance of a Ca^{2+}-activated K^+ channel in MDCK cells and to consider its possible role in net K^+ and Cl^- transport. In recent years considerable attention has focussed on the molecular mechanism by which a number of organs involved in K^+ homeostasis; such as mammalian distal nephron segments and mammalian descending colon (Garcia-Filho et al, 1980; Schultz, 1981; Fromm & Schultz, 1981; Moreto et al, 1981), regulate net transepithelial K^+ movements. In mammalian colon the mechanism of K^+ secretion is still unclear. There is evidence that K^+ secretion is active and via a transcellular route (Yorio & Bentley, 1977; Wills & Biago, 1980), but there is also evidence to suggest that K^+ secretion is paracellular and driven by the favourable electrical gradient across the epithelium (Frizzell et al, 1976; Fromm & Schultz, 1981; Schultz, 1981) and is abolished under short circuit conditions. Recently Moreto

and colleagues (1981) suggested that the involvement of a Ca^{2+} dependent increase in the apical membrane permeability may account for at least part of the secretagogue stimulated K^+ efflux across in vitro colon. For MDCK cells no net K^+ movements are observed under short circuit conditions (see Chapter 4), and a paracellular route for K^+ movements would appear unlikely due to the low permeability of the shunt pathway for K^+. It is possible however that net K^+ secretion across the apical membrane may occur in a similar manner to that proposed by Moreto and co-workers (1981): K^+ is accumulated into the cell above its electrochemical potential by the $(Na^+ + K^+)$-ATPase located upon the basal-lateral membranes. Stimulation of a Ca^{2+}-dependent K^+ permeability at either membrane face by secretagogues results in a net loss of K^+ into both bathing solutions. K^+ lost into the basal bathing solution is then re-cycled. K^+ loss across the apical cell membrane is potential sensitive.

In chapters 3 and 4 I proposed that adrenaline stimulated Cl^- secretion in high resistance MDCK cells was compatible with the proposed models of Cl secretion by natural epithelia. According to this model Cl^- secretion is the result of an electroneutral coupled Na + Cl accumulation across the basal-lateral border followed by a passive electrochemically downhill exit step across the apical cell membrane (see fig 3.2). Under short circuit conditions Cl^- secretion is electrogenic, the current being carried across the apical membrane by the net basal to apical Cl^- flux. To complete the electrical circuit an equal amount of current must be carried across the basal-lateral cell membrane. Since the entry step for Cl^- consists primarily of a coupled electroneutral Na + Cl uptake then clearly the current must be carried by some other

mechanism. There are two likely candidates: firstly the Na^+-pump which is electrogenic, and secondly, current could be carried by K^+ efflux across the basal-lateral cell membrane. This raises the possibility that there are two rate limiting steps for Cl^- secretion under short circuit conditions: the current carrying capacity of the basal-lateral cell membrane, and the electrochemical gradient for Cl^- across the apical cell membrane. Potentiation of Cl^- secretion by alpha-receptors may be the result of a modification of either of these parameters.

TABLE 5.1

The effects of apical and basal additions of adrenaline (100uM), ATP (100uM) and A23187 (10uM) upon the fractional ^{86}Rb efflux across the apical and basal-lateral cell borders. The fractional ^{86}Rb loss is expressed as a ratio of the efflux rates at T = 4 minutes against T = 0 minutes (T_4/T_0). The numbers in parentheses are the number of observations for each condition. All drugs were added at T = 0 minutes.

Significantly different from control response:

a = non significant

b = $p < 0.01$

c = $p < 0.001$

Condition	T_4/T_0 Apical	T_4/T_0 Basal
Control	0.8 ± 0.1 (8)	0.9 ± 0.1 (8)
100uM Adrenaline (apical addition)	0.9 ± 0.2^{a} (3)	1.0 ± 0.1^{a} (4)
100um Adrenaline (basal addition)	1.5 ± 0.1^{b} (6)	2.28 ± 0.3^{c} (6)
10 uM A23187 (apical addition)	1.5 ± 0.1^{b} (4)	2.58 ± 0.1^{c} (4)
10 uM A23187 (basal addition)	0.8 ± 0.2^{a} (4)	0.8 ± 0.1^{a} (4)
100 uM ATP (apical addition)	1.8 ± 0.1^{c} (4)	3.3 ± 0.2^{c} (4)
100 uM ATP (basal addition)	1.9 ± 0.2^{c} (4)	2.7 ± 0.2^{c} (4)

TABLE 5.2

The effect of various adrenoreceptor agonists and antagonists upon fractional ^{86}Rb loss. The results are expressed as the ratio of fractional ^{86}Rb effluxes two minutes following adrenaline stimulation compared to Rb efflux immediately prior to adrenaline addition (T_2/T_0). Results are expressed as the mean $\pm$ S.E. The number of separate determinations is indicated in parentheses.

Significantly different from 0.1mM adrenaline

a = $p < 0.01$

Condition	Response T_2/T_0	P v Control
Control	0.984 ± 0.02 (9)	-
0.1mM Adrenaline	2.24 ± 0.25 (10)	0.001
0.1mM Isoprenaline	1.05 ± 0.04 (6)	N.S.
0.1mM Oxymetazoline	0.98 ± 0.06 (6)	N.S.
0.1mM Phenylephrine	1.44 ± 0.04 (4)	0.001
0.1mM Adrenaline + 1um Propranolol	3.75 ± 0.42 (5)	0.001
0.1mM Adrenaline + 5uM Phentolamine	0.96 ± 0.09 (5)	N.S.

TABLE 5.3

The effect of a Ca-free bathing solution and of various pharmacological agents upon the adrenaline stimulated increase in total fractional ^{86}Rb efflux from subconfluent monolayers of MDCK cells grown upon plastic petri-dishes. The fractional ^{86}Rb loss is expressed as a ratio of the efflux rate at T = 2 minutes against T = 0 minutes (T_2/T_0). The effect of the pharmacological agents was tested in normal Krebs' by including the appropriate drug in the adrenaline containing Krebs'. The effects of various conditions upon non-stimulated ^{86}Rb effluxes was also examined, in separate control experiments, where the ratio (T_2/T_0) of fractional ^{86}Rb effluxes refer to each experimental condition. The figures in parentheses refer to the number of observations for each condition. Adrenaline or the various drugs were added at T = 0 minutes.

Significantly different from control response :

a = not significant

b = $p < 0.05$

c = $p < 0.01$

d = $p < 0.001$

Experimental condition	Effect upon basal ^{86}Rb efflux T_2/T_0	Control response to 100uM adrenaline T_2/T_0	Test response to 100uM adrenaline T_2/T_0
Ca - free + 2mM EGTA	0.88 ± 0.01 (3)	2.07 ± 0.20 (12)	1.17 ± 0.11[c] (7)
1mM Quinine	1.14 ± 0.30 (6)	2.39 ± 0.05 (3)	1.05 ± 0.08[d] (3)
1mM Ouabain	1.13 ± 0.05 (6)	2.41 ± 0.27 (9)	1.68 ± 0.16[b] (11)
25mM tetra-ethylammonium	0.73 ± 0.12 (4)	1.68 ± 0.04 (4)	1.21 ± 0.09[c] (4)
1mM isomethyl butylxanthine (IBMX)	1.07 ± 0.06 (3)	2.10 ± 0.21 (4)	1.99 ± 0.08[a] (4)
25nM Apamin	0.92 ± 0.04 (2)	1.76 ± 0.42 (4)	1.72 ± 0.08[a] (4)
0.1mM Furosemide	0.64 ± 0.04 (8)	2.41 ± 0.27 (9)	2.63 ± 0.11[a] (5)

		Response to 20uM A23187	Test response to 20uM A23187
1mM Quinine		5.18 ± 0.24 (4)	1.36 ± 0.11[c] (4)

TABLE 5.4

The effects of a nominally Ca^{2+}-free bathing solution and various pharmacological agents upon the ATP stimulated ^{86}Rb efflux from subconfluent monolayers of MDCK cells grown upon plastic petri-dishes. Fractional ^{86}Rb efflux is expressed as the ratio of efflux rates at T = 2 against T = 0. All drugs with the exception of quinine were added at T = 0, quinine was added at T = -6 minutes. The numbers in parentheses are the number of observations for each condition.

Condition	Control response to 100uM ATP. T_2/T_0	Test response to 100uM ATP. T_2/T_0	P v Control
'Ca^{2+} - free'	5.45 ± 0.21 (6)	2.73 ± 0.26 (8)	0.001
Quinine (1mM)	5.86 ± 0.26 (4)	2.09 ± 0.15 (4)	0.001
T.E.A. (25mM)	5.46 ± 0.24 (5)	1.4 ± 0.2 (5)	0.001
Ouabain (1mM)	6.14 ± 0.3 (4)	7.42 ± 0.23 (4)	0.02
Theophylline (1mM)	3.75 ± 0.18 (4)	4.69 ± 0.27 (4)	0.05
IBMX (1mM)	3.04 ± 0.18 (7)	3.56 ± 0.21 (7)	N.S.
Furosemide (100uM)	6.14 ± 0.3 (4)	6.57 ± 0.53 (4)	N.S.
Apamin (25nM)	3.04 ± 0.06 (4)	4.09 ± 0.46 (4)	N.S.

TABLE 5.5

The effect of 100uM adrenaline upon the magnitude of the three components of K^+ influx. The results are expressed as a percentage of the total K^+ influx in the absence of 100uM adrenaline. The results are the means $\pm$ S.E. of six separate determinations. Total control K^+ influx was 3.62 $\pm$ 0.34 (S.E. n = 12) mmol/l cell water per minute. The ouabain sensitive component was determined using 10^{-3} M ouabain, whilst the furosemide sensitive component was determined in ouabain containing solutions with 10^{-4} M furosemide.

Significantly different from control values:

a = non significant

Components of Flux	Adrenaline uM	% of Control Influx
Total influx	0	100 ± 8.3
	100	114 ± 15.6[a]
Ouabain sensitive component	0	45.8 ± 5.2
	100	47.9 ± 6.2[a]
Furosemide sensitive component	0	35.4 ± 8.3
	100	29.1 ± 11.4[a]
Ouabain + furosemide insensitive component	0	25 ± 5.2
	100	22.9 ± 3.1[a]

TABLE 5.6

The effect of 100uM adrenaline upon intracellular K^+ ion content of subconfluent cell monolayers of MDCK cells grown upon plastic petri dishes. The results are expressed as the mean $\pm$ S.E.M of at least 6 observations from two seperate experiments. 100uM adrenaline was added at t=0 minutes.

Significantly different from control values:

a = < 0.02

b = < 0.01

c = < 0.001

Time (min)	Intracellular K^+ uEq. x 10^6 cells	
	Control	100uM Adrenaline
2	355.4 ± 6.4 (7)	298 ± 9.4 [b] (7)
4	367.6 ± 7.8 (7)	266.6 ± 6.0 [c] (7)
6	373.6 ± 5.8 (7)	309 ± 6.4 [c] (7)
8	378.4 ± 3.2 (7)	330.6 ± 6.4 [a] (6)

TABLE 5.7

A comparison of the actions of adrenaline, exogenous ATP and A23187 upon the fractional ^{86}Rb efflux from subconfluent monolayers of either high resistance (strain I) or low resistance (strain II) MDCK cells. ^{86}Rb effluxes are expressed as the ratio of ^{86}Rb efflux at T = 2 minutes against T = 0 minutes. The various drugs were added at T = 0 minutes. The numbers in parentheses are the number of observations for each condition.

Significantly different from control values :

a = non significant

b = $p < 0.01$

c = $p < 0.001$

	^{86}Rb Efflux T_2/T_0		
Condition	Strain I	Strain II	p value
Control	0.97 ± 0.01 (7)	0.91 ± 0.06 (7)	N.S.
100uM Adrenaline	1.93 ± 0.15[c] (8)	0.82 ± 0.03[a] (7)	0.001
100uM ATP	4.33 ± 0.60[c] (7)	3.18 ± 0.27 (7)	N.S.
20uM A23187	3.58 ± 0.14[c] (3)	2.49 ± 0.14[c] (4)	0.01
20uM A23187 (Ca-free)	1.64 ± 0.04[c] (3)	1.67 ± 0.04[c] (4)	N.S.

TABLE 5.8

The effect of nominally Ca^{2+}-free media, voltage clamping and various inhibitors of Ca^{2+} activated K^{+} permeabilities across the apical and basal aspects of confluent epithelial preparations. ^{86}Rb efflux is expressed as the ratio of fractional ^{86}Rb loss at T = 4 minutes against T = 0 minutes. Figures in parentheses are the number of observations of each condition. Adrenaline was added to the basal bathing solution in each instance at T = 0 minutes.

Significantly different from control values:

a = non significant

b = $p \leq 0.05$

c = $p < 0.01$

Significantly different from adrenaline (open circuit) values:

d = non significant

e = $p < 0.05$

f = $p < 0.01$

g = $0.1 > p > 0.05$

Condition	T_4/T_0 Apical	T_4/T_0 Basal
Control	0.82 ± 0.06 (10)	0.84 ± 0.05 (10)
100uM Adrenaline open circuit	1.76 ± 0.27^{c} (24)	1.89 ± 0.2^{c} (24)
100uM Adrenaline voltage clamped PD = OmV	$1.1 \pm 0.09^{b,g}$ (8)	$1.96 \pm 0.12^{c,d}$ (8)
100uM Adrenaline Ca free + 2mM EGTA	$1.02 \pm 0.1^{a,f}$ (8)	$0.94 \pm 0.11^{a,e}$ (8)
100uM Adrenaline + 1mM Quinine (apical)	$1.23 \pm 0.28^{a,d}$ (8)	$0.94 \pm 0.06^{a,e}$ (8)
100uM Adrenaline + 1mM Quinine (basal)	$1.18 \pm 0.23^{a,d}$ (8)	$0.66 \pm 0.07^{a,e}$ (8)
100uM Adrenaline + 25mM TEA (apical)	$0.65 \pm 0.2^{a,e}$ (3)	$1.1 \pm 0.2^{a,g}$ (3)
100uM Adrenaline + 25mM TEA (basal)	$0.97 \pm 0.1^{a,e}$ (3)	$1.2 \pm 0.2^{a,g}$ (3)

Fig 5.1 Action of adrenaline upon fractional ^{86}Rb efflux across the apical and basal-lateral cell aspects of MDCK cell monolayers.

Abscissa: fractional ^{86}Rb efflux (x100). Ordinate: time (miuntes). Epithelial cell monolayers were pre-loaded with ^{86}Rb for 5 hours, washed x4 with Krebs and mounted into Ussing chambers. Fractional ^{86}Rb efflux across the apical and basal-lateral cell membranes was measured, simultaneously, at 2 minute intervals (see Methods for detailed description). The data is from a single representative experiment, grouped data is shown in table 5.1. Adrenaline was added to the appropriate bathing solution at t = 0 minutes. (O., △ , □) efflux across the apical cell aspects. (● , ▲ , ■) efflux across the basal-lateral cell aspects. (O , ●) addition of 100uM adrenaline to the basal-lateral bathing solution. (△ , ▲) addition of 100uM adrenaline to the apical bathing solution. (□ , ■) efflux minus adrenaline.

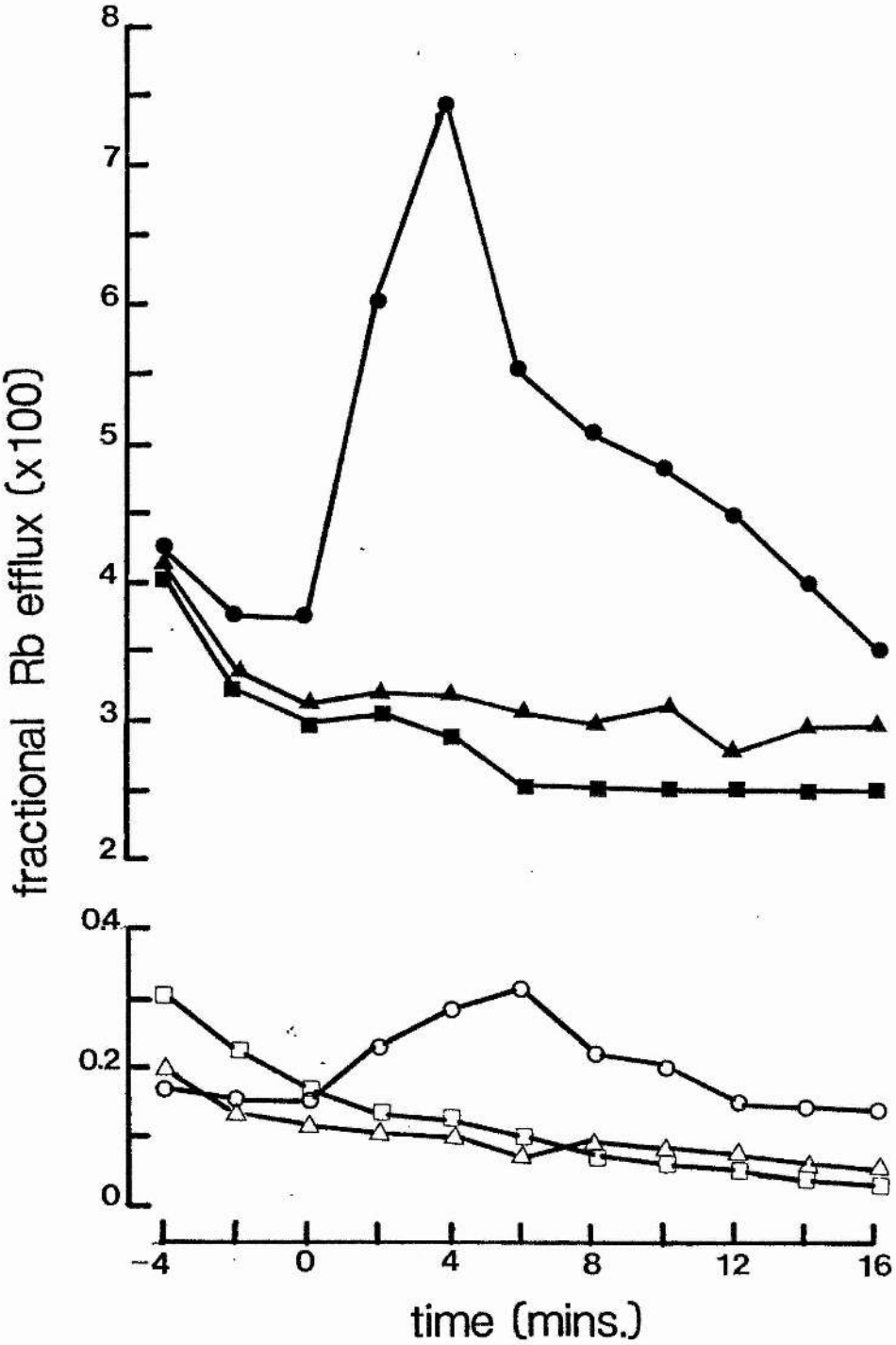

fractional Rb efflux (x100)
8
7
6
5
4
3
2
0.4
0.2
0
-4
0
4
8
12
16
time (mins.)

Fig 5.2 Kinetic analysis of ^{86}Rb efflux across the basal-lateral cell membranes of confluent MDCK cell monolayers.

Abscissa: $\log_e$ counts per minute. Ordinate: time (minutes). ^{86}Rb loss across the basal-lateral cell membrane of confluent cell monolayers, grown upon permeable filter supports comprises loss of isotope from two compartments. ^{86}Rb efflux can be approximated by the sum of two exponentials of rate constants 0.19 min^{-1} and 4.9×10^{-2} min^{-1}. The inset shows a regression line for the first exponential which obeys the equation:

$$y = -0.085(x) + 0.75$$

The solid line in the main diagram represents the least-squares regression line and obeys the equation:

$$y = -0.021(x) + 3.79$$

The slope is significantly different to zero ($P < 0.001$) and the correlation coefficient was 0.926 ($P < 0.001$). Each datum is the mean $\pm$ SEM of 4 observations.

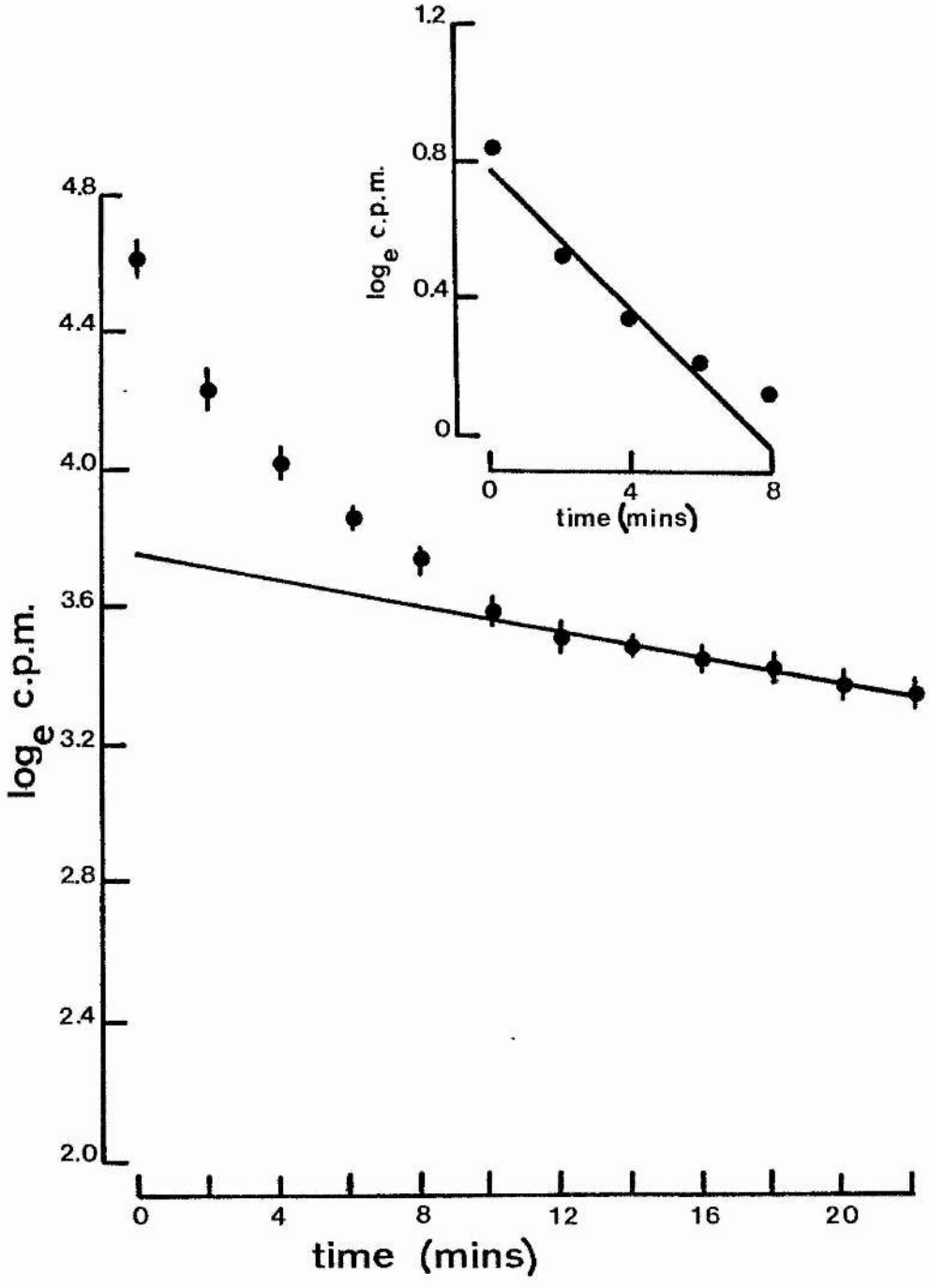

1.2
0.8
0.4
0
log$_e$ c.p.m.
0
4
8
time (mins)
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
log$_e$ c.p.m.
0
4
8
12
16
20
time (mins)

Fig 5.3 Effect of exogenous ATP upon fractional ^{86}Rb loss across the apical and basal-lateral cell aspects of MDCK cell monolayers.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: time (minutes). Epithelial cell monolayers were preloaded with ^{86}Rb for 5 hours, washed x4 with Krebs and mounted into Ussing chambers. Fractional ^{86}Rb efflux across both cell membranes was determined simulatneously, at two minute intervals. ATP was added to the appropriate bathing solution at t = 0 minutes. The data is taken from one representative experiment. Grouped data is shown in table 5.1. (△, ○) efflux across the apical cell membrane. (▲, ●) efflux across the basal-lateral membrane. (△, ▲) addition of ATP to the apical bathing solution. (○, ●) addition of ATP to the basal-lateral bathing solution. The ATP concentration used was 100 uM.

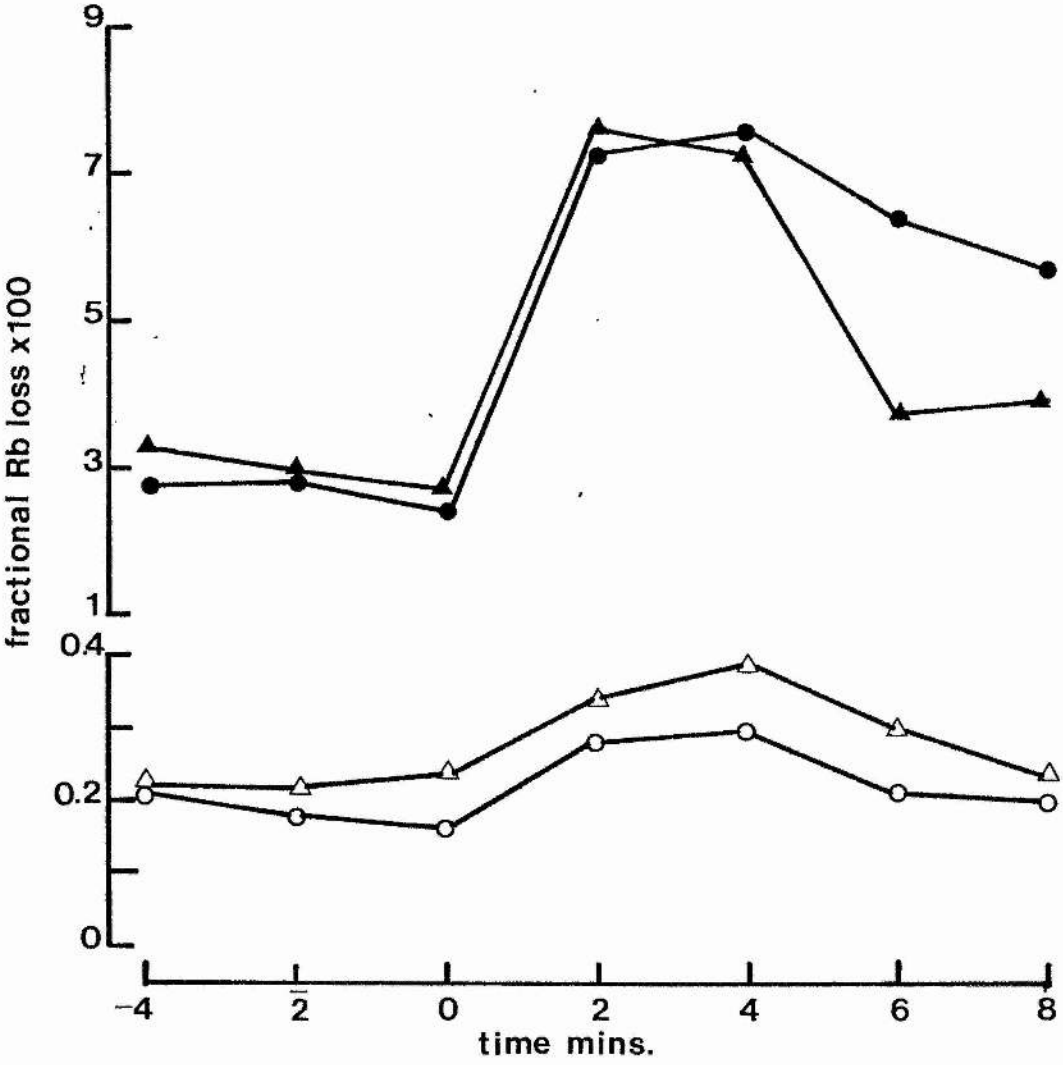

9
7
5
3
1
0.4
0.2
0
fractional Rb loss x100
−4
2
0
2
4
6
8
time mins.

Fig 5.4 Action of 10 uM A23187 upon fractional ^{86}Rb efflux across the apical and basal-lateral cell aspects of MDCK cell monolayers.

Abscissa: fractional ^{86}Rb efflux (x100). Ordinate: time (minutes). Epithelial cell monolayers were pre-loaded with ^{86}Rb for 5 hours, washed x4 in Krebs and mounted into Ussing chambers. Fractional ^{86}Rb efflux across the apical and basal-lateral cell membranes was measured, simultaneously, at 2 minute intervals.

The data is from one representative experiment, grouped data is shown in table 5.1. A23187 was added to the appropriate bathing solution at t = 0 minutes. (△, ○) efflux across the apical cell membranes. (▲, ●) efflux across the basal-lateral cell membranes. (△, ▲) addition of 10 uM A23187 to the apical bathing solution. (○ , ●) addition of 10 uM A23187 to the basal-lateral bathing solution.

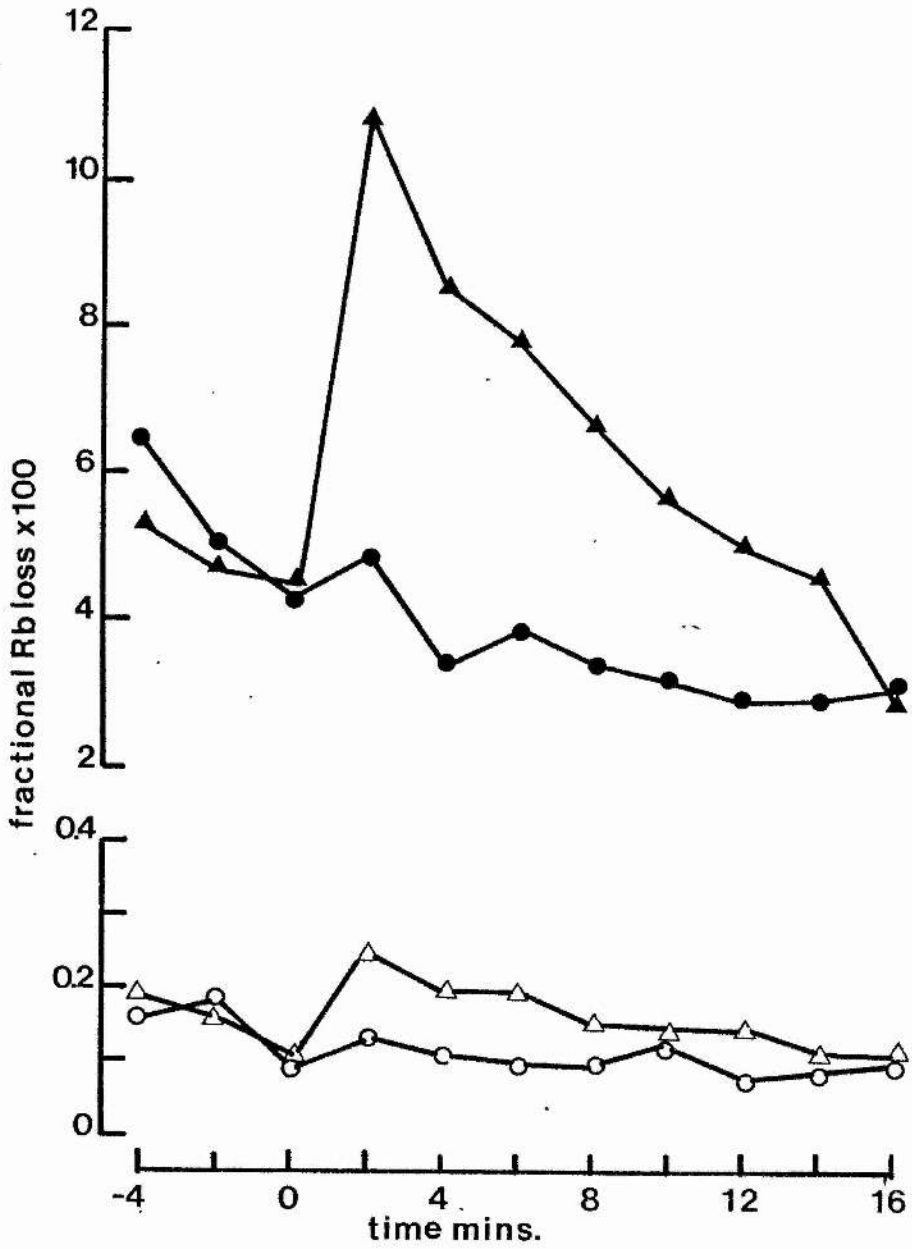

12
10
8
6
4
2
fractional Rb loss x100
0.4
0.2
0
-4
0
4
8
12
16
time mins.

Fig 5.5 Kinetic analysis of ^{86}Rb efflux from subconfluent cell monolayers.

Abscissa: $\log_e$ counts per minute. Ordinate: time (minutes).

After an initial two minute period ^{86}Rb efflux from subconfluent cell monolayers of MDCK cells comprises of loss of isotope from a single compartment. ^{86}Rb efflux can be approximated by a single exponential with a rate constant of 4.83×10^{-2} min^{-1}. The solid line represents the least-squares regression line and obeys the equation :

$$y = -0.022(x) + 3.89$$

The slope of the line was significantly different from zero $p<0.001$ and the correlation coefficient was 0.996. The results are expressed as mean $\pm$ SEM of 4 separate determinations.

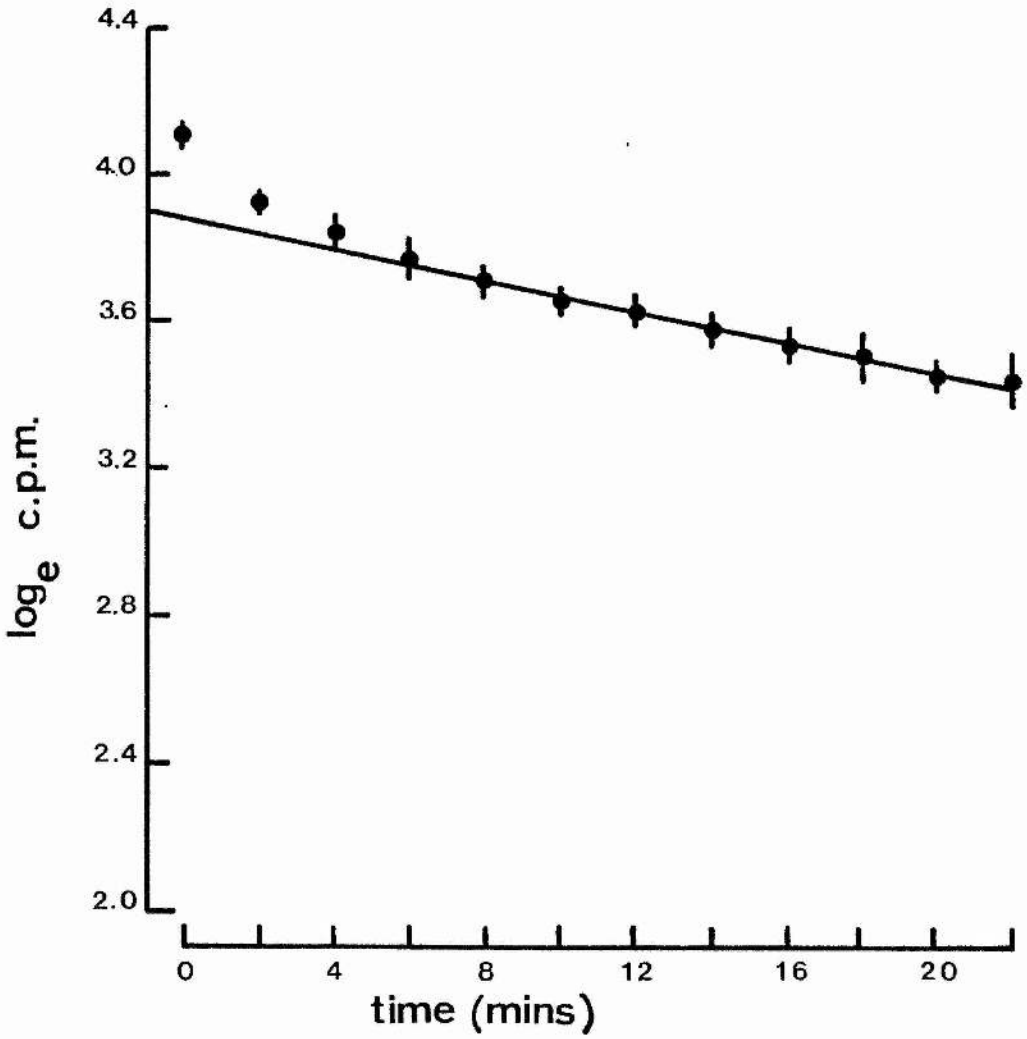

4.4
4.0
3.6
3.2
2.8
2.4
2.0
log$_e$ c.p.m.
0
4
8
12
16
20
time (mins)

Fig 5.6 Effect of adrenaline upon fractional ^{86}Rb efflux from subconfluent monolayers of MDCK cells.

Abscissa: fractional ^{86}Rb efflux (x100). Ordinate: time (minutes). Subconfluent monolayers of MDCK cells were grown upon plastic petri dishes, and preloaded for 3 hours with ^{86}Rb. Fractional ^{86}Rb efflux was measured at 2 minute intervals. Adrenaline (100 uM) was added to the wash solution at t = 0 minutes. Each datum point is the mean $\pm$ SEM of 20 observations. (●) control efflux, (▲) plus 100 uM adrenaline.

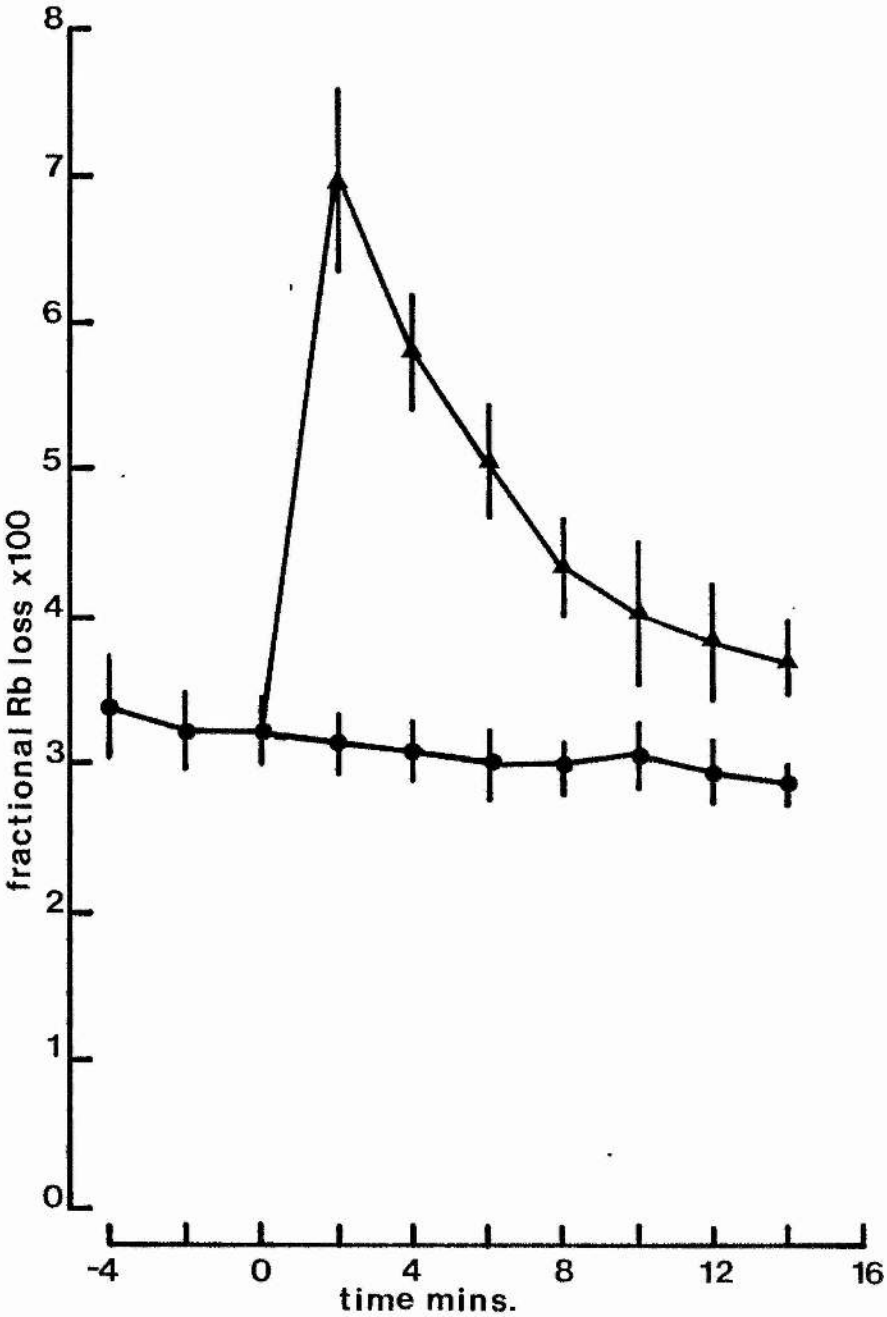

8
7
6
5
4
3
2
1
0
fractional Rb loss x100
-4
0
4
8
12
16
time mins.

Fig 5.7 Effect of exogenous ATP upon fractional ^{86}Rb efflux from subconfluent monolayers of MDCK cells.

Abscissa: fractional ^{86}Rb efflux (x100). Ordinate: time (minutes). Subconfluent monolayers were grown upon plastic petri dihes and preloaded with ^{86}Rb for 3 hours. Fractional ^{86}Rb efflux was measured at 2 minute intervals. ATP (100 uM) was added at t = 0 minutes. Each datum point is the mean $\pm$ SEM of 22 observations. (●) control efflux, (▲) plus 100 uM ATP.

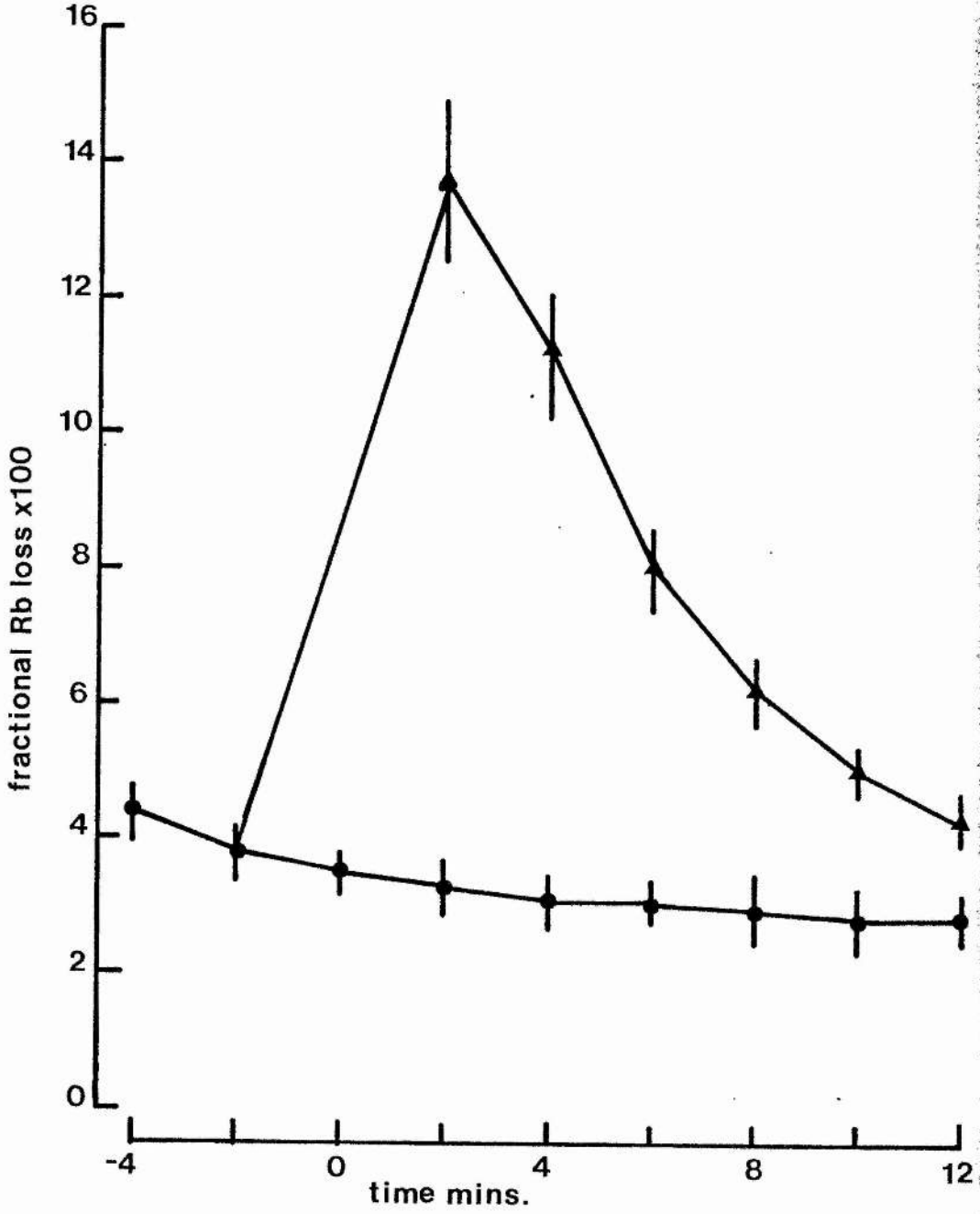

16
14
12
10
8
6
4
2
0
fractional Rb loss x100
-4
0
4
8
12
time mins.

Fig 5.8 Effect of A23187 upon fractional ^{86}Rb efflux from subconfluent monolayers of MDCK cells.

Abscissa: fractional ^{86}Rb efflux. Ordinate: time (minutes). Subconfluent cell monolayers were preloaded with ^{86}Rb for 3 hours. Fractional ^{86}Rb efflux was measured at two minute intervals. A23187 was added at time = 0 minutes. Each datum point is the mean $\pm$ SEM of 5 observations. (●) control efflux, (▲) plus 20 uM A23187.

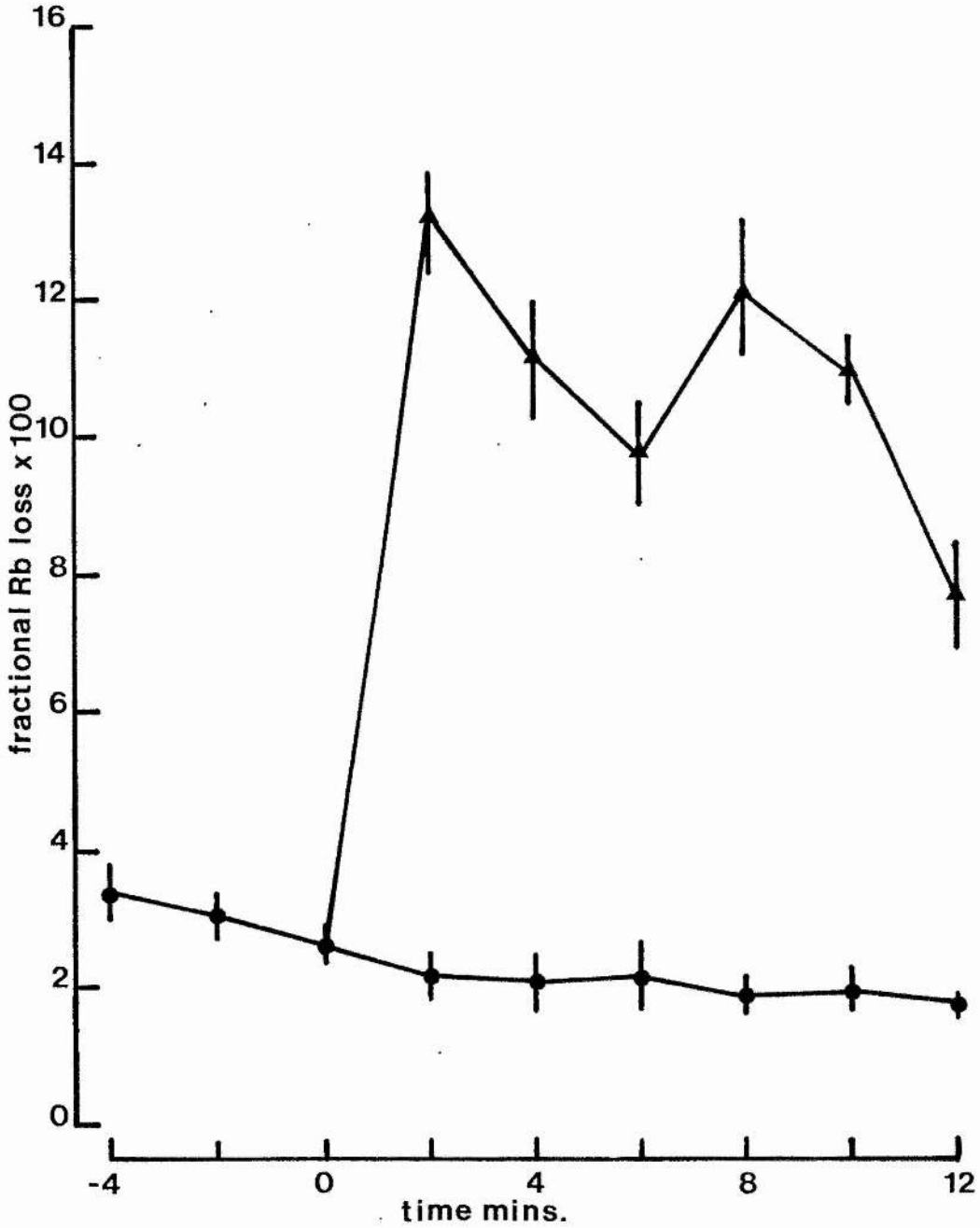

16
14
12
10
8
6
4
2
0
fractional Rb loss x 100
-4
0
4
8
12
time mins.

Fig 5.9 Log dose response curve for the ability of adrenaline to stimulate fractional ^{86}Rb loss from MDCK cells.

Abscissa: ratio of the fractional ^{86}Rb efflux 2 minutes post-adrenaline addition (T_2) against the fractional ^{86}Rb loss immediately prior to adrenaline addition (T_0). Ordinate: Log_{10} adrenaline concentration. The ability of adrenaline to stimulate ^{86}Rb loss from subconfluent cell monolayers, grown upon plastic petri dishes was studied. Half maximal stimulation of ^{86}Rb efflux was observed at $9.1 \pm 0.2 \times 10^{-7}M$ adrenaline. This value was calculated from a Probit analysis of the data presented above. The curve was fitted by eye. Each datum is the mean and SEM of 3 observations.

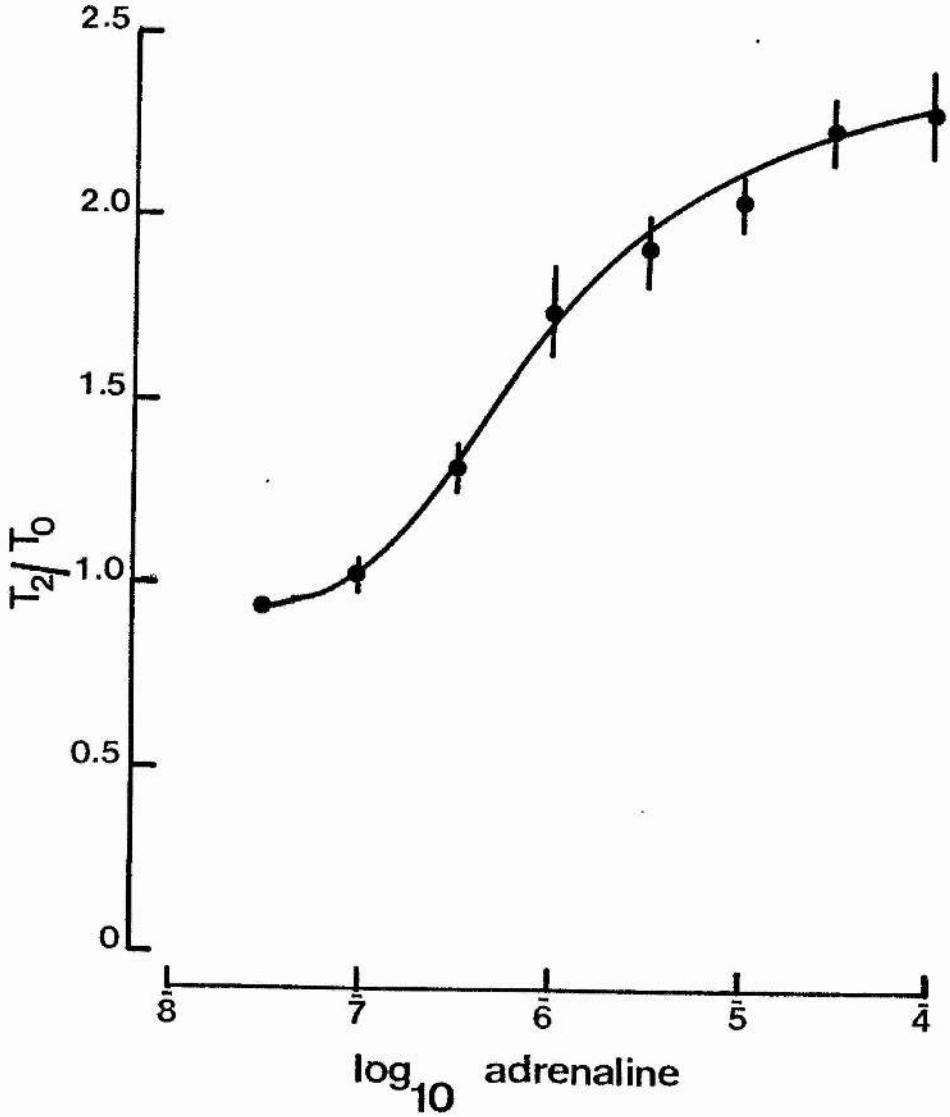

2.5
2.0
1.5
1.0
0.5
0
T_2/T_0
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ adrenaline

Fig 5.10 Log dose response curve for the ability of ATP to stimulate fractional ^{86}Rb efflux from MDCK cells.

Abscissa: the ratio of fractional ^{86}Rb loss two minutes post-ATP addition against the rate of fractional ^{86}Rb efflux immediately prior to ATP addition (T_2/T_0). Ordinate: Log_{10} ATP concentration. The ability of ATP to stimulate fractional ^{86}Rb loss from MDCK cells was tested upon subconfluent cell monolayers. Half maximal stimulation of ^{86}Rb loss was observed at $4.4 \pm 0.4 \times 10^{-6}$M ATP. This value was calculated from a Probit analysis of the data presented in the fig above. Each datum is a single determination of efflux.

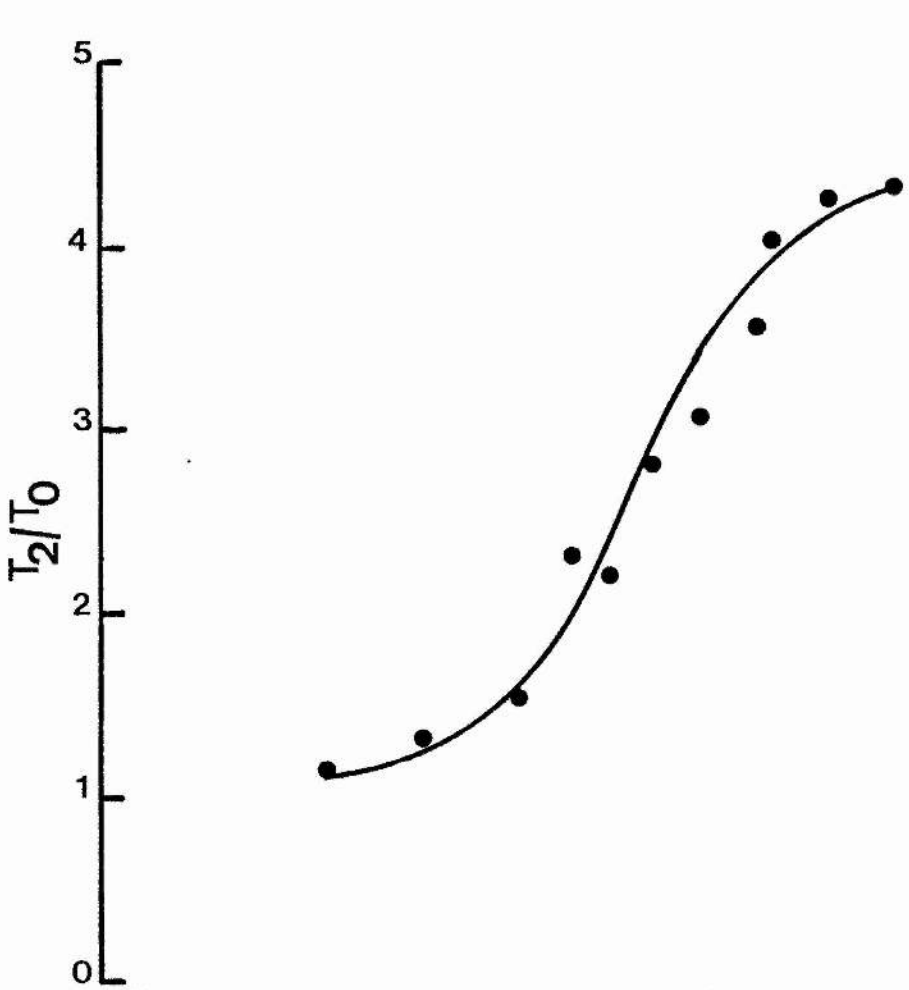

5
4
3
2
1
0
T_2/T_0
$\bar{8}$
$\bar{7}$
$\bar{6}$
$\bar{5}$
$\bar{4}$
$\log_{10}$ ATP

Fig 5.11 Dose dependency of the actions of A23187.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: time (minutes). The ability of different concentrations of ionophore A23187 to stimulate ^{86}Rb efflux was tested upon subconfluent monolayers of MDCK cells grown upon plastic petri dishes. A23187 was added at t = 0 minutes. Each concentration represents the mean data from two separate cell monolayers.

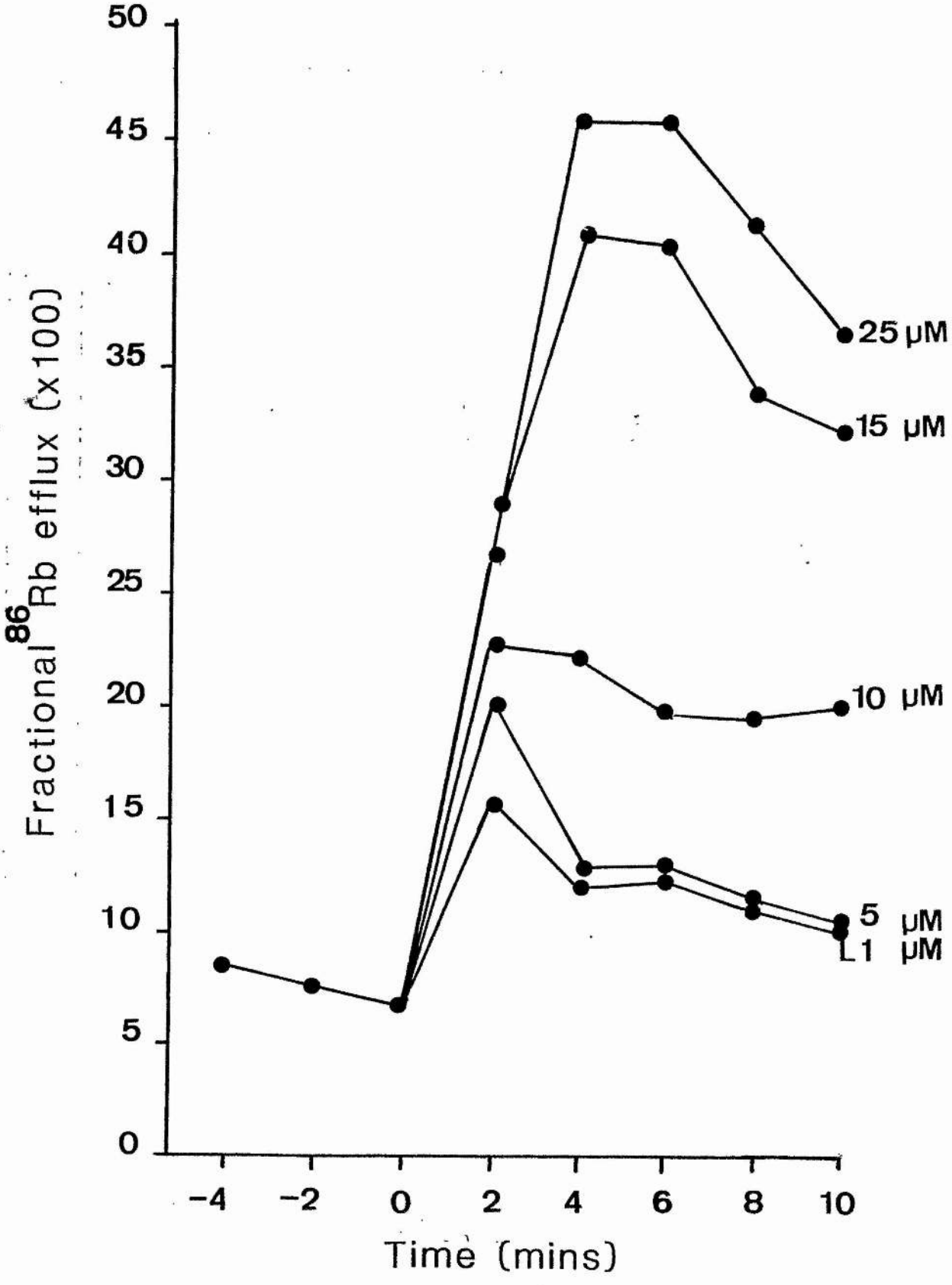

50
45
40
35
30
25
20
15
10
5
0
Fractional ^{86}Rb efflux (x100)
25 µM
15 µM
10 µM
5 µM
1 µM
-4
-2
0
2
4
6
8
10
Time (mins)

Fig 5.12 Desensitisation of adrenaline stimulated ^{86}Rb efflux in the continued presence of adrenaline.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: time (minutes). A six-minute exposure to 100 uM adrenaline (●) is followed by a six-minute drug-free period (○), after which time either 100 uM adrenaline (●) is re-applied, or the cells are challenged with 20 uM A23187 (■). A control response to 20 uM A23187 was obtained upon separate subconfluent cell monolayers from the same batch (▲). Each datum point is the mean of at least 4 observations. Standard error of the mean was less than 5%.

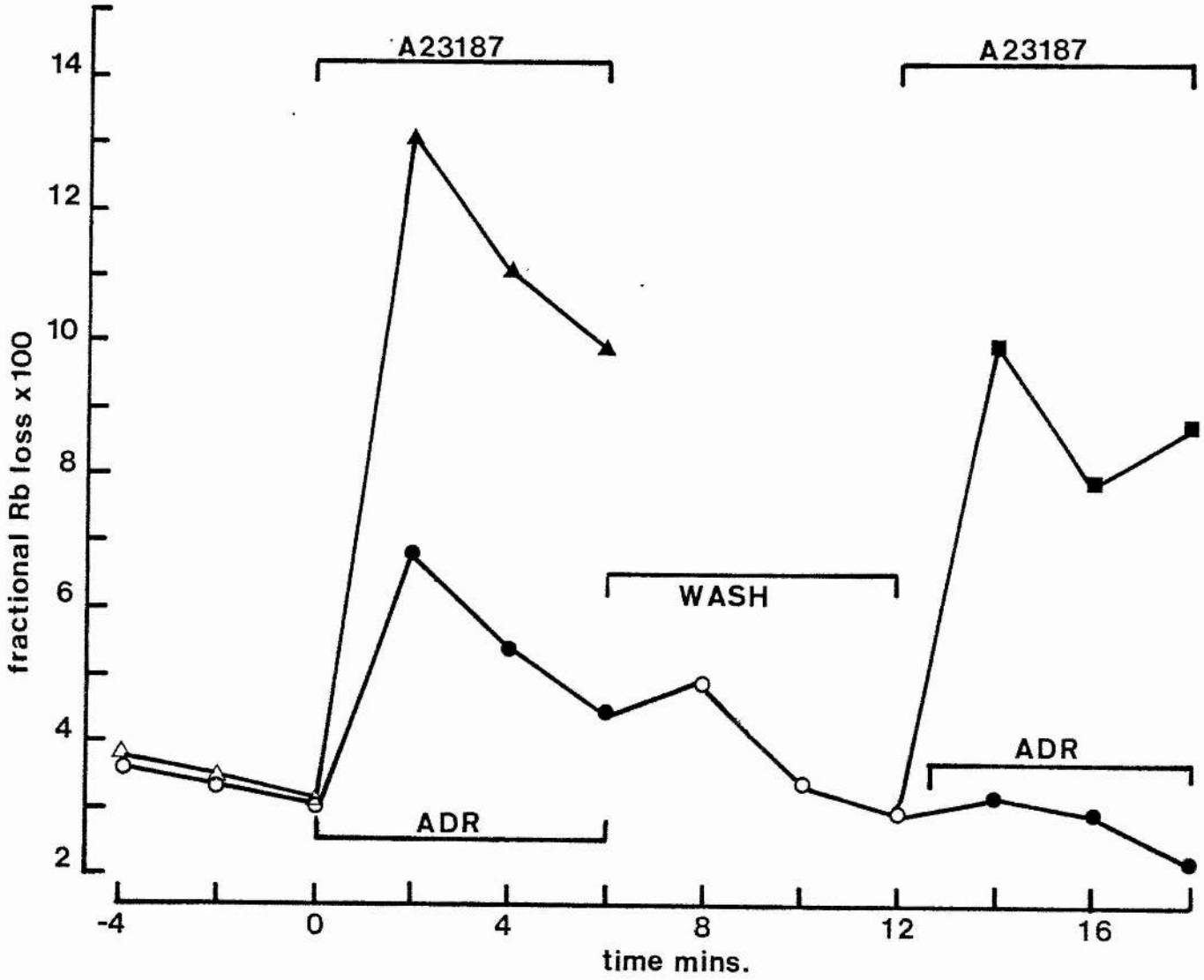

A23187
A23187
WASH
ADR
ADR
fractional Rb loss x100
14
12
10
8
6
4
2
-4
0
4
8
12
16
time mins.

Fig 5.13 Cross receptor desensitisation of the Ca^{2+} activated K^{+} permeability.

Abscissa: fractional ^{86}Rb loss (x100). Ordinate: time (minutes). To differentiate between a receptor mediated desensitisation of the response and a Ca^{2+}-dependent inactivation of the K^{+} permeability, the ability of adrenaline (100 uM) to stimulate ^{86}Rb efflux from subconfluent monolayers of MDCK cells was tested after exposure of the monolayers to 100 uM ATP. At t = 0 minutes cell monolayers were challenged with 100 uM ATP, after an 8 minute incubation; 100 uM adrenaline was included into the experimental solution and fractional ^{86}Rb efflux measured for another 10 minutes. A control response to 100 uM adrenaline was measured upon separate subconfluent monolayers from the same batch. (○) control fractional ^{86}Rb efflux, (●) fractional ^{86}Rb efflux in the presence of 100 uM ATP, (▲) fractional ^{86}Rb efflux in the presence of 100 uM ATP and 100 uM adrenaline, (■) fractional ^{86}Rb efflux in the presence of adrenaline. Each datum is the mean $\pm$ SEM of 3 observations. In some cases the SEM was smaller than the symbol size.

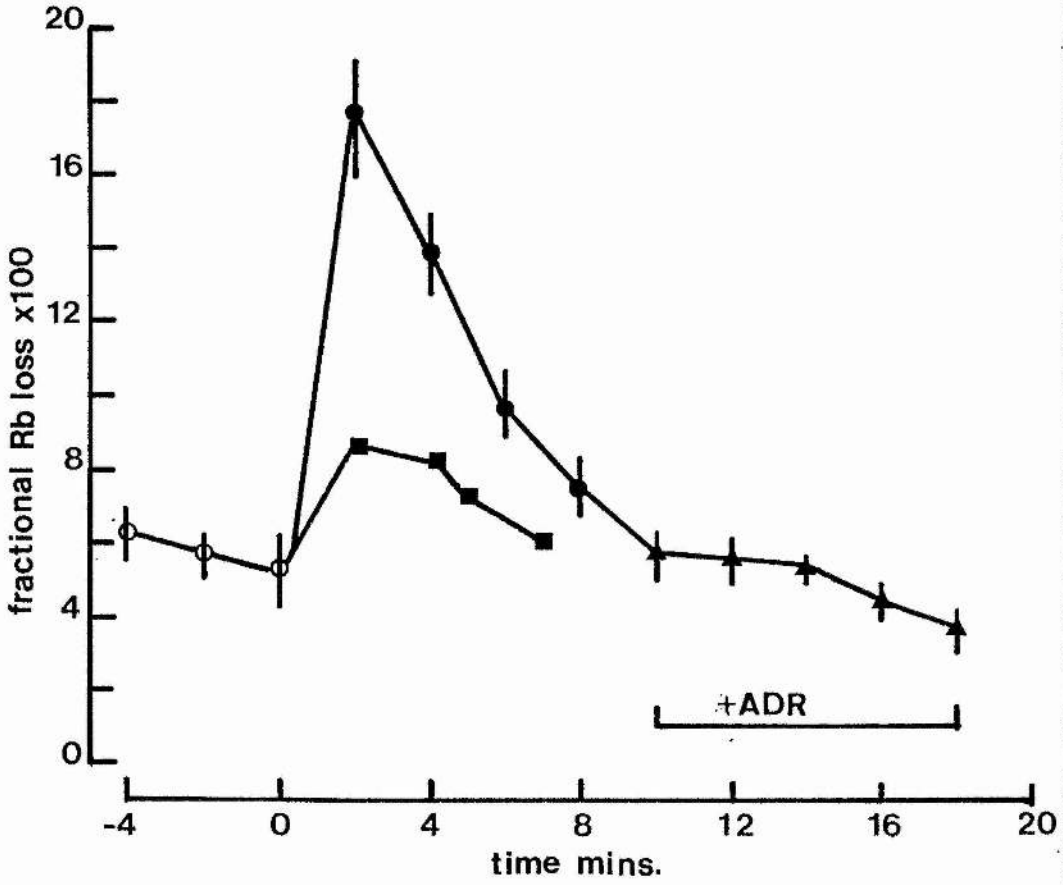
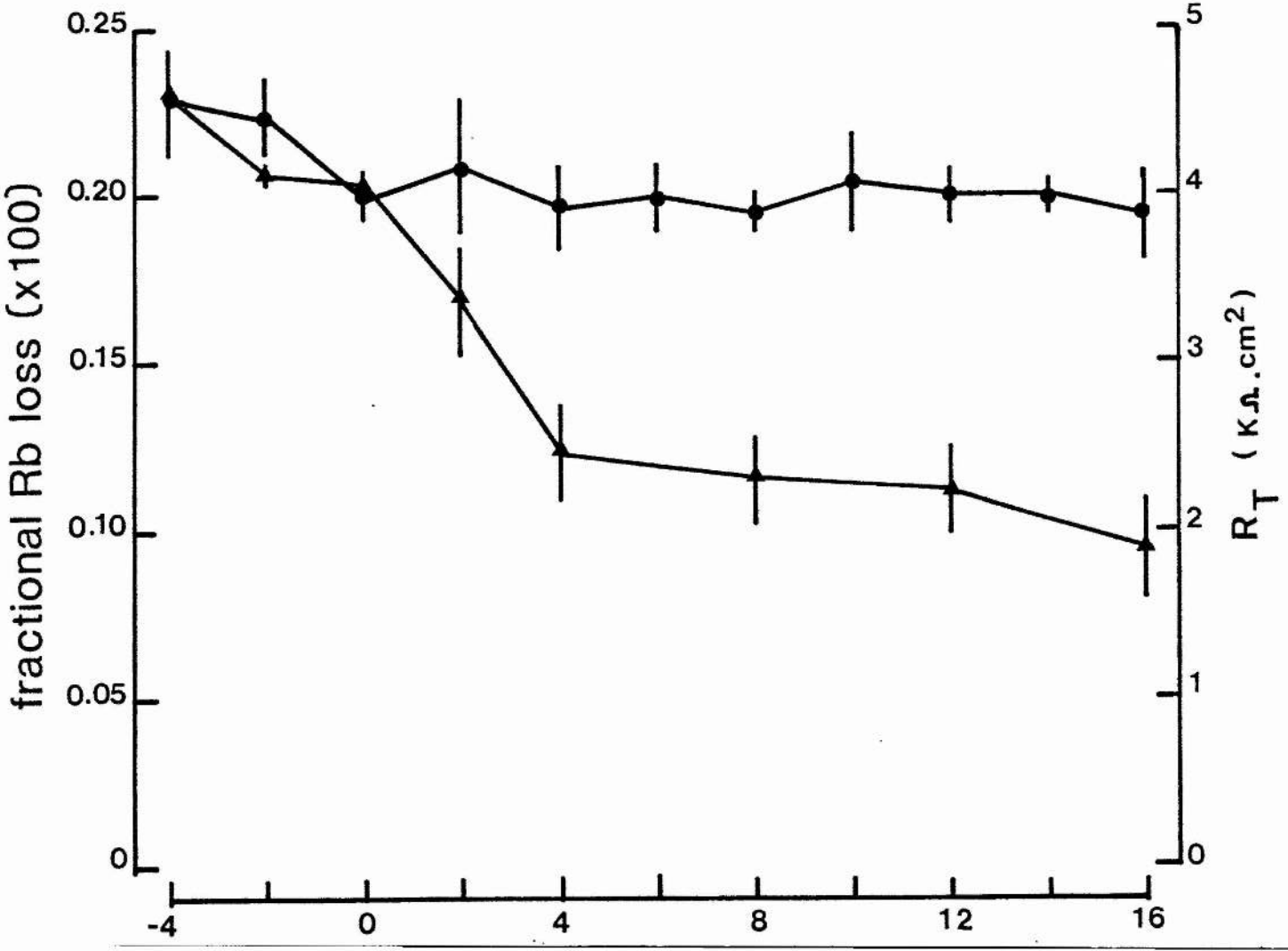

20
16
12
8
4
0
fractional Rb loss x100
+ADR
-4
0
4
8
12
16
20
time mins.

Fig 5.14 The effect of varying transepithelial resistance upon the steady-state fractional ^{86}Rb loss across the apical membrane.

Abscissa: right; fractional ^{86}Rb loss (x100), left; transepithelial electrical resistance ($k\Omega.cm^2$). Ordinate: time (minutes).

To test the contribution a paracellular shunt played in the appearance of ^{86}Rb in the apical bathing solution, transepithelial resistance, and hence the magnitude of the paracellular shunt, was varied by incubating confluent cell monolayers, grown upon permeable filter supports, in a Ca^{2+}-free (+ 2mM EDTA) bathing media and simultaneously measuring ^{86}Rb loss into the apical bathing media. Despite large changes in transepithelial resistance (▲), there was no signifcant increase in fractional ^{86}Rb loss into the apical bathing solution (●). Each datum is the mean ± SEM of 4 observations.


fractional Rb loss (x100)
0.25
0.20
0.15
0.10
0.05
0
5
4
3
2
1
0
R_T (KΩ.cm^2)
-4
0
4
8
12
16

CHAPTER 6

CONCLUDING REMARKS

In the last few years cultured epithelial cell systems have been increasingly employed in the study of epithelial polarity and function (see recent reviews by Handler (1980a) and Wright (1981). However, it must be realised that the usefulness of a cultured cell-line in these studies is governed by two main considerations: Firstly, the cell-line must still remain differentiated in culture and exhibit the morphological and biochemical asymmetry of its plasma membranes that is a characteristic feature of all epithelia, and perhaps more importantly, the retention of a polarised morphology should also be accompanied by an expression of some of the physiological and transport functions of its tissue of origin. The second consideration is that a cultured cell system is used in such a way as to exploit its inherent advantages over an equivalent _in vivo_ or _in vitro_ experimental preparation. A well characterised tissue culture model epithelium is the Madin and Darby canine kidney (MDCK) cell-line. Initially derived from a homogenate of whole dog kidney (Madin & Darby, 1958; Gausch _et al_, 1966) the MDCK cell-line has been extensively used in studies of the development and maintenance of epithelial polarity (see below). When grown upon glass, plastic or permeable filter substrates, MDCK cells exhibit a typical epithelial morphology: apical brush border, apical cell-cell tight junctions and lateral interspaces. When grown upon a permeable substrate and mounted in Ussing chambers, confluent monolayers of MDCK cells exhibit a mean transepithelial electrical resistance of either 80-100 $\Omega \cdot cm^2$ or 3-4 $k\Omega \cdot cm^2$ depending on cell strain (see introduction and also Barker & Simmons, 1981).

Despite a large literature upon the retention of polarity by MDCK cells, comparitively little was known about the physiological and transporting

properties of MDCK cell monolayers. The aim of this work was therefore to address the question of whether MDCK cells expressed any of the physiological or transporting properties normally associated with renal epithelia in particular, or natural epithelia in general? The results of these studies have been presented in the preceding three chapters and are summarised below.

When MDCK cells (strain I) were grown upon permeable millipore filter supports and mounted into Ussing type chambers they exhibited a mean transepithelial resistance of $3.5 k\Omega.cm^2$ and supported a small transepithelial potential of 1.2 mV (basal-lateral positive). However this result was thought to be an underestimate of the true resistance and potential due to crush damage incurred during mounting. Subsequent measures of potential and resistance from monolayers mounted in a new chamber, specifically designed to alleviate crush damage, confirmed this view since the mean transepithelial resistance of $7.9 k\Omega.cm^2$ and potential of 5.9 mV was close to that theoretically predicted for monolayers mounted free from crush damage (Barker & Simmons, 1981). In both epithelial preparations the monolayers supported a small (0.34 and 0.74 $uAmps.cm^{-2}$ respectively) short circuit current in agreement with the small magnitude of the net fluxes of Na^+, K^+ and Cl^-.

In culture MDCK cells have retained a number of hormone receptors normally associated with renal epithelia (Richardson et al, 1981). Addition of adrenaline to the basal-lateral bathing solution stimulated an increase in short circuit current, due to both an increased transepithelial potential difference and a decreased transmonolayer resistance. In contrast, addition of adrenaline to the apical bathing solution was without effect upon short circuit current, implying the receptors

mediating the response were located exclusively upon the basal-lateral cell surface. A pharmacological characterisation of the receptors mediating the response suggests that the adrenaline stimulated short circuit current was the result of stimulation of beta-adrenoreceptors, although the response was potentiated by activation of alpha-receptors, also located upon the basal-lateral cell aspects. Measurement of tracer Na^+, K^+ and Cl^- fluxes in the presence of adrenaline demonstrated that the short circuit current response was the result of a stimulation of net Cl^- secretion from the apical to the basal-lateral cell surface. The characteristics of Cl^- secretion in MDCK cells bore strong resemblances to the properties of Cl^- secretory mechanisms in other epithelia (Frizzell et al, 1979), and could be explained in terms of existing models of epithelial Cl^- secretion (see fig 3.2). Thus it is postulated that Cl^- is accumulated into MDCK cells above its electrochemical equilibrium by an electroneutral coupled $Na^+ + Cl^-$ transport mechanism located at the basal-lateral cell membrane. Although the exact nature of this mechanism is a matter of debate (Frizzell et al, 1979; Liedtke & Hopfer, 1982a, 1982b) in a number of epithelia, there is strong evidence to suggest that in MDCK cells Cl^- accumulation across the basal-lateral cell membrane is achieved by a diuretic sensitive electroneutral $Na^+ + K^+ + Cl^-$ cotransport system: both cotransport and Cl^- secretion exhibit similar pharmacological sensitivities; sensitive to inhibition by the loop diuretics Furosemide, piretanide and bumetamide and also by phloretin and ethacrynic acid, but insensitive to inhibition by SITS and amiloride. Cl^- secretion and the diuretic sensitive cotransport system also display an absolute requirement for the presence of both Na^+ and Cl^- in the basal-lateral bathing solution: Na^+ could

be replaced by Li^+ but not by choline, and Cl^- could be replaced by Br^- but not by NO_3^-, I^-, SCN^- or isethiocyanate. Diuretic sensitive cotransport has been implicated in the Cl^- entry step in a number of Cl^- transporting epithelia (Eveloff et al, 1978, 1980; Greger & Schlatter, 1981). The second step in this model is a hormone induced apical membrane permeability for Cl^- through which Cl^- exits down a favourable electrochemical gradient. Evidence for this step in MDCK cells is the observation that adrenaline decreases transmonolayer resistance, a decrease which is still seen when Cl^- secretion is inhibited by furosemide. However, no direct evidence has been presented to localise this conductance increase to the apical membrane.

Measurement of K^+ influx and K^+ efflux across the apical and basal-lateral cell membrane demonstrated that the apical cell membrane was relatively impermeable to K^+, similar to a number of 'tight' epithelia, and that K^+ fluxes were dominated by flux across the basal-lateral cell membrane. K^+ influx across the basal-lateral membrane consisted of three major components: a ouabain sensitive component, a diuretic sensitive component, and a component insensitive to inhibition by ouabain and furosemide. In contrast, influx across the apical membrane consisted of one component, a ouabain and furosemide insensitive flux. The diuretic sensitive component of flux was Cl^- dependent and was mediated by the $Na^+ + K^+ + Cl^-$ cotransport system located on the basal-lateral membrane of MDCK cells (Aiton et al, 1982).

K^+ efflux across the basal-lateral cell aspects was also sensitive to inhibition by furosemide and furosemide plus ouabain, indicating that the loop diuretic sensitive cotransport system is primarily involved in $K^+ : K^+$ exchange. Ouabain caused a time dependent increase in the rate

of K^+ loss across the basal-lateral cell membrane which was furosemide sensitive.

Addition of adrenaline, exogenous ATP or Ca^{2+} ionophore A23187 stimulated a transient increase in K^+ loss across both the apical and basal-lateral cell membranes, although flux across the basal-lateral membrane was of greatest quantitative importance. The secretagogue induced increase in fractional K^+ loss was a net flux since: (1) adrenaline had no effect upon either the magnitude or distribution of K^+ influx among its three components, and (2) adrenaline caused a net loss of cellular K^+ as measured by flame photometry of tissue extracts. The actions of the secretagogues were of high affinity and dose dependent: half maximal stimulation of fractional K^+ loss was observed at 9.1×10^{-7}M adrenaline and with 4.4×10^{-6}M ATP. Analysis of the receptor type mediating the adrenaline stimulated K^+ loss was consistent with the response being mediated by an alpha-adrenoreceptor.

The effects of adrenaline, ATP and ionophore A23187, were abolished in a Ca^{2+}-free media. The pharmacological sensitivity of the secretagogue stimulated K^+ efflux is similar to Ca^{2+} dependent K^+ permeabilities in other tissues, notably red blood cells and colon, including inhibition by quinine and tetraethylammonium chloride and insensitivity to the bee venom, toxin apamin. Although the physiological role of a Ca^{2+} dependent K^+ permeability in MDCK cells has yet to be elucidated, two possible roles come to mind: firstly, stimulation of K^+ efflux by adrenaline may potentiate adrenaline stimulated Cl^- secretion, either by providing a more favourable electrochemical gradient for downhill Cl^- movement across the apical membrane or, under short circuit conditions, carrying current across the basal-lateral membrane. A second possible

role for the Ca^{2+} dependent K^+ efflux may be in epithelial K^+ secretion, as has been recently proposed by Moreto (1981).

The results presented in this thesis identify and characterise three major transport systems in high resistance MDCK cells that are also found in natural epithelia: (1) A catecholamine stimulated Cl^- secretory mechanism compatable with the current models of Cl^- secretion in epithelia (Frizzell et al, 1979). (2) A $Na^+ + K^+ + Cl^-$ cotransport system similar to those identified in a wide range of other tissues including epithelia (Chipperfield, 1981; Geck et al, 1981; Greger & Schlatter, 1981). (3) A Ca^{2+} dependent K^+ permeability at both the apical and basal-lateral cell membranes with properties similar to Ca^{2+} dependent K^+ conductances in red blood cells (Lew & Ferreira, 1978), glandular tissue (Putney, 1979) and mammalian colon (Moreto et al, 1981). These results, coupled with polarised morphology of MDCK cells, strongly support its use as a model system with which to study epithelial cell function.

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